THE
BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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THE INFLUENCE OF TEMPERATURE UPON THE COMBINATION OF OXYGEN WITH THE BLOOD OF TROUT [1, 2]

LAURENCE IRVING, EDGAR C. BLACK AND VIRGINIA SAFFORD

(From the Edward Martin Biological Laboratory, Swarthmore College, Swarthmore, Pennsylvania)

The air breathed by all species of mammals is the same in composition, and the affinity of their blood for oxygen is also much alike. But the water in which fish live differs greatly in oxygen saturation in different places and seasons, and this variability is particularly conspicuous in bodies of fresh water in temperate regions. Under these circumstances it is not surprising to find that the blood of various species of fish varies greatly in affinity for oxygen. Krogh and Leitch (1919) first remarked upon the differences among the eel, carp, plaice, cod, and trout, and regarded these differences in affinity for oxygen as evidence for the adaptation of the blood to the conditions in which each species lived. But only a few species were examined and these were quite dissimilar in form and habit as well as in respect to the habitat which they occupied.

The catfish, carp, bowfin, and sucker were found by Black (1940) to have blood with different oxygen dissociation curves, which were nevertheless related as if in one family. These freshwater fish are similar in form and are all of free swimming habit. Since their respiratory requirements and their physical systems for providing oxygen are much alike, it is reasonable to consider that large differences in the properties of the blood of these species indicate the suitability of the blood for respiration in different environments.

We have now examined the blood of eight species of freshwater fish and find that the affinity for oxygen varies greatly among the species. Furthermore, as Black (1940) pointed out for four of the species, the

[1] We wish to acknowledge the kind assistance of Mr. C. O. Hayford, Superintendent of the New Jersey State Fish Hatchery at Hackettstown, in enabling us conveniently to secure the blood from the trout used in these experiments.

[2] The expenses of this investigation were in part provided by a grant from the Rockefeller Foundation.

effect of CO_2 upon oxygen combination is also quite different in the species. Table I shows the usual oxygen affinity and the effect of CO_2 in the blood of these eight species. These characteristics would fit respiration under quite different conditions.

These properties of the blood do not depict its natural suitability for the transport of oxygen because there is no allowance for the variety

TABLE I

Oxygen affinity and effect of CO_2 in blood of freshwater fish at 15° (Jordan, 1929)

Species	P CO_2 at ½ saturation		Limit of CO_2 effect	
	P CO_2 = 1–2 mm.	P CO_2 = 10 mm.	Percentage HbO_2	
Common catfish *Ameiurus nebulosus*	1.4	5		(Black, 1940)
Bowfin *Amia calva*	4	9		(Black, 1940)
Carp *Carpiodes cyprinus*	5	8	85	(Black, 1940)
Yellow perch *Perca flavescens*			77	(Irving, unpublished)
Common sucker *Catostomus commersonnii*	12	43	71	(Black, 1940)
Chain pickerel *Esox niger*			53	(Irving, unpublished)
Rainbow trout *Trutta iridea*	18	35	52	(Irving, Black, and Safford—this paper)
Brown trout *Trutta trutta*	17	39	52	(Irving, Black, and Safford—this paper)
Brook trout *Salvelinus fontinalis*	17	42	52	(Irving, Black, and Safford—this paper)
Atlantic salmon (freshwater) *Salmo salar*	19	35	57	(Irving, 1939)
Lake trout *Cristivomer namaycush*			40	(Irving, unpublished)

of temperatures in the natural habitats of the fish. In lakes in temperate regions the temperature of a stratum of water may differ sharply from the temperature above and below, and the seasonal changes are rapid and large. The influence of temperature upon the oxygenation of mammalian blood is such that at 20° it would be 95 per cent saturated with oxygen by a pressure of 45 mm., while at 37° the pressure required is about 100 mm. (Brown and Hill, 1923). According to the figures

given for the blood of the skate (Dill et al, 1932), the hemoglobin would be 95 per cent saturated by 40 mm. pressure of oxygen at 15°, but the same saturation would require 170 mm. at 25°. At the higher temperature part of the efficacy of the hemoglobin for oxygen transport would be lost even in water which was saturated with air. The blood of the eel would be 95 per cent saturated with oxygen by pressures of 12, 25, and 71 mm. at 5°, 17°, and 30° respectively (Kawamoto, 1929). From general considerations concerning the nature of hemoglobin, as well as from these two examples, it is to be expected that the function of oxygen transport in fish blood is considerably influenced by the temperature at which it occurs.

The combination of oxygen with the blood of fish is peculiarly sensitive to carbon dioxide, as Krogh and Leitch (1919) first observed. Even at 700 mm. pressure of oxygen the hemoglobin of the blood of the tautog will not become saturated with oxygen in the presence of carbon dioxide (Root and Irving, 1940). In this respect the CO_2 effect in fish blood differs from the Bohr effect produced by CO_2 in mammalian blood. In the practical absence of CO_2 the hemoglobin of fish blood is saturated with oxygen at 150 mm. pressure. With increasing pressures of CO_2, oxygen saturation falls off until no further reduction of oxygen saturation is secured beyond 60 mm. pressure of CO_2 (Root, Irving, and Black, 1939). At the limit of the CO_2 effect on the blood of trout only about 50 per cent of the hemoglobin can be oxygenated.

The limit of the CO_2 effect is quite different in various species of fish. A number of freshwater fish which we have examined clearly show the variation in the limit of the CO_2 effect in the blood of several species (Table I, column 3). The differences in CO_2 sensitivity of the blood and the influence which the CO_2 effect would exert upon conditions essential for respiratory transport, however, have only been determined at 15°.

The limit of the CO_2 effect in trout blood changes with temperature, as is shown by the observations recorded in Fig. 1. These determinations were made by measuring the percentage of saturation of the hemoglobin with oxygen at a pressure of 150 mm. and CO_2 at 65 mm. or more. The blood samples were secured from a number of brook, brown, and rainbow trout. The limit of the CO_2 effect falls at a lower level of oxygen saturation with increasing temperature up to 25°. Above 25° the blood cannot be saturated by oxygen at 150 mm. pressure even in the absence of CO_2.

The influence which temperature exerts upon the CO_2 effect shows that the effect of CO_2 upon affinity for O_2 must be considered in making

a description of the blood of fish which will be useful in designating its suitability for the performance of its natural function of oxygen transport.

Material Used

For examining the effect of temperature upon oxygen affinity and the CO_2 effect we have selected three common and related species of freshwater fish; brook trout, *Salvelinus fontinalis;* brown trout, *Trutta*

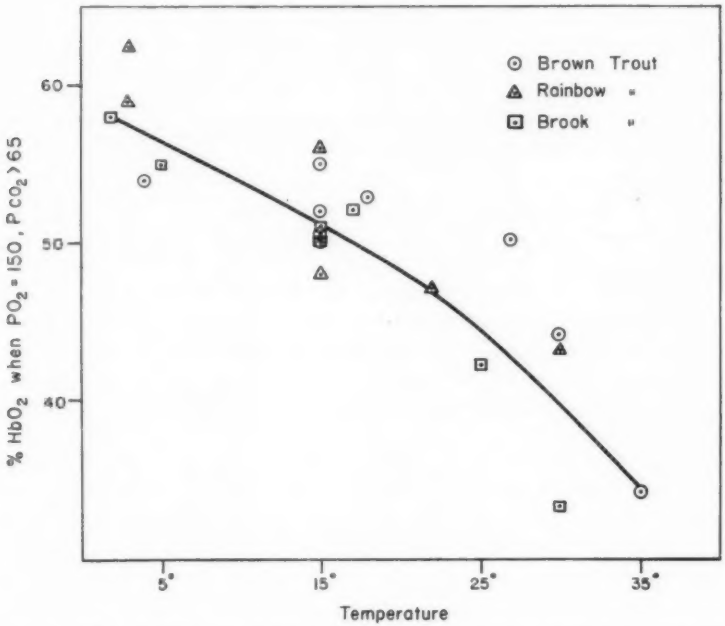

Fig. 1. Limit of the effect of CO_2 upon oxygenation of hemoglobin in trout blood.

trutta; and rainbow trout, *Trutta iridea.* These fish live under similar conditions and are alike in activity. They were raised in the same water at the New Jersey State Hatchery at Hackettstown. All had received the same sort of food, and each species had been raised from a homogeneous and selected stock. If they were as different in form and habits as the toadfish and the tautog, all of the physical components of the respiratory mechanism would obviously be different, and a comparison of the species on the basis of the properties of blood alone would

not be justified. But since these three species of trout are so much alike, against the background of general similarity of essential respiratory devices either similarity or difference in the properties of the blood for respiratory transport may acquire significance for the eventual picture of specific respiratory adaptation.

Methods

Male fish of from 1½ to 2½ pounds weight were bled by syringe from the heart, yielding from 5 to 20 ml. per fish. Coagulation was prevented by liquid heparin (Connaught Laboratory), the blood was iced, and analysis was begun about five hours after drawing and completed within the next fourteen hours. About five of the eighty fish used died but the others showed no ill effects from bleeding.

The blood was equilibrated for more than 20 minutes with suitable gas mixtures. Half-milliliter samples were withdrawn and analyzed according to the method of Van Slyke and Neill (1924). Extraction of the blood in the apparatus was complete after six minutes. Samples of the equilibrated gases were analyzed in the Haldane apparatus. Hematocrit measurements were made with a high speed (6000 r.p.m.) centrifuge.

Each sample of blood was analyzed after equilibration at from two to five temperatures. If the procedure was prolonged, final results were checked by repetition of an early equilibration. It was possible to keep the blood without change at 30° for at least an hour, but after a short time at 35° the oxygen capacity was not restored by equilibration at 15°.

Mixtures of the blood of several fish gave results comparable with the blood of single fish. Blood samples from 23 brook trout, 23 brown trout, and 34 rainbow trout were examined on five separate days for each species. Each species was examined in March, October, November, and December. No fish were used soon after stripping the sperm for breeding, and there was no apparent seasonal change in the blood.

The temperature of the natural spring water at Hackettstown was uniform at about 12°, but in the pools it fell as low as about 6° without noticeably altering the blood. With a good flow of water in the hatchery and stock selected for a number of years conditions are especially favorable for a degree of uniformity that has not been encountered in our experience with several other species of wild fish.

Oxygen Capacity

When equilibrated with air at temperatures below 25° and with pressure of CO_2 less than 2 mm., the hemoglobin is saturated with oxy-

TABLE II

Oxygen capacity

Species	Number of fish	HbO_2 ml. O_2 per 100 ml. blood	Cell volume ml. O_2 per 100 ml. blood
Brook trout *Salvelinus fontinalis*	23	11.7 (11.0–13.9)	40
Brown trout *Trutta trutta*	23	12.2 (11.1–14.4)	35
Rainbow trout *Trutta iridea*	34	13.8 (13.0–15.0)	43

gen. The oxygen combined with hemoglobin was determined by subtracting from the total oxygen in the blood the dissolved oxygen, which was estimated from the solubility coefficients for oxygen dissolved in

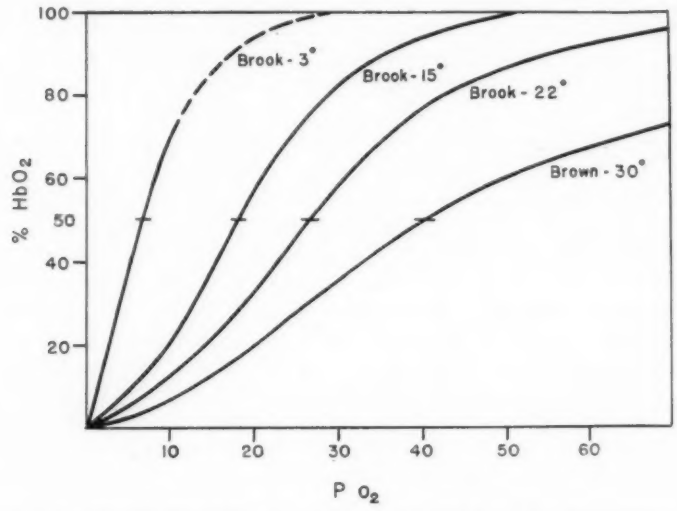

FIG. 2. Oxygen dissociation curves of trout blood at various temperatures.

mammalian blood (Peters and Van Slyke, 1935). The amount of oxygen combined with the saturated hemoglobin is designated as oxygen capacity. Its value is shown for the three species in Table II. Since the samples were frequently mixed from several fish, the variations in oxygen capacity are not the limits of those which may occur among individuals. The oxygen capacities were slightly larger than in the cat-

fish, bowfin, carp, and sucker (Black, 1940), and distinctly larger than in many of the marine fish examined by Root (1931).

Oxygen Dissociation Curves

In our experience the oxygen affinity of fish hemoglobin is not perceptibly diminished by CO_2 unless the tension exceeds 2 mm. The

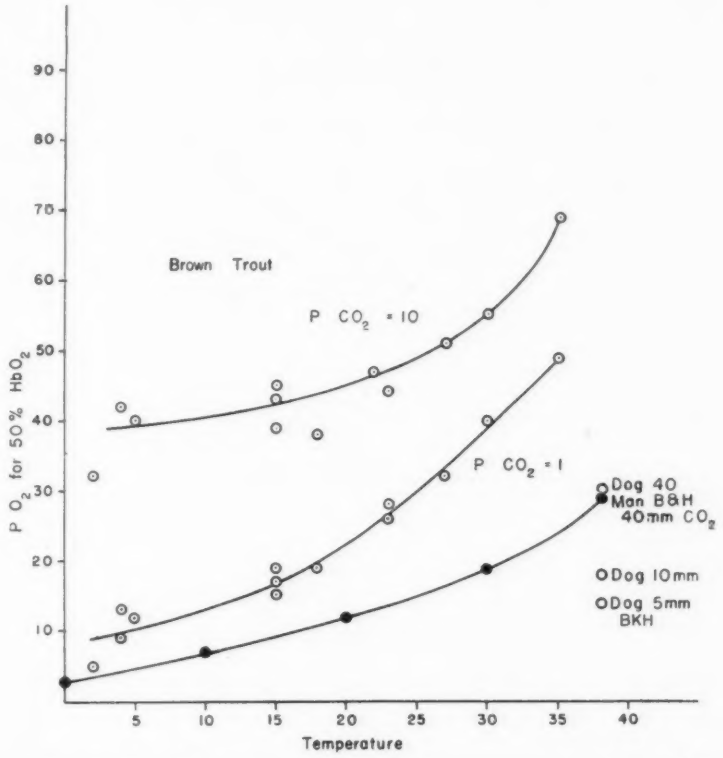

Fig. 3. The effect of temperature upon the pressure of oxygen needed to half-saturate blood of brown trout at CO_2 tensions 1 and 10 mm. Human blood (Brown and Hill, 1923) and some points for dogs' blood (Bohr, Hasselbalch, and Krogh, 1904) are shown for comparison.

oxygen dissociation curves shown in Fig. 2 were made with blood having tensions of CO_2 less than 2 mm., drawing the best lines through points from several samples of mixed blood of the brook trout. One sample

of mixed blood from two brown trout was examined over the range of oxygen pressures at 30°. Considering that there are individual variations and that the construction of a number of complete curves for each species at each temperature is not practical, it may be said that these are representative curves, and that one family of similar curves depicts the oxygen affinity of the blood of all three species at various temperatures.

In the gills of trout, loading naturally occurs with tensions of CO_2

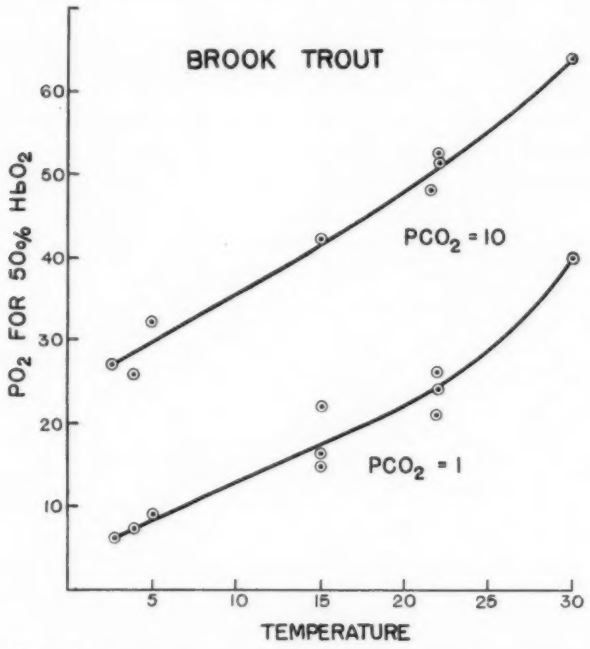

FIG. 4. The effect of temperature upon the pressure of oxygen necessary to half-saturate blood of brook trout at CO_2 tensions 1 and 10 mm.

in the water which are less than 2 mm. (Ferguson and Black, 1940). In the arterial blood of rainbow trout the tension of CO_2 is about as low, and so the curves represent the condition of the blood when loading with oxygen occurs in the gills. At 30° the blood could not be saturated with the oxygen pressure of the air, but at 25° the hemoglobin could reach its full saturation.

At temperatures above 15° it is easy to see that the curves are *S*-shaped. At lower temperatures the deviation is too small to be dem-

onstrated with certainty. At any temperature the curve is sufficiently close to a straight line so that a single determination of the oxygen content and tension of blood between 40 per cent and 60 per cent saturation establishes within 2 mm. the point where the curve cuts half saturation. In this manner it was possible by determining the PO_2 for half saturation of the hemoglobin in one lot of blood at a number of tempera-

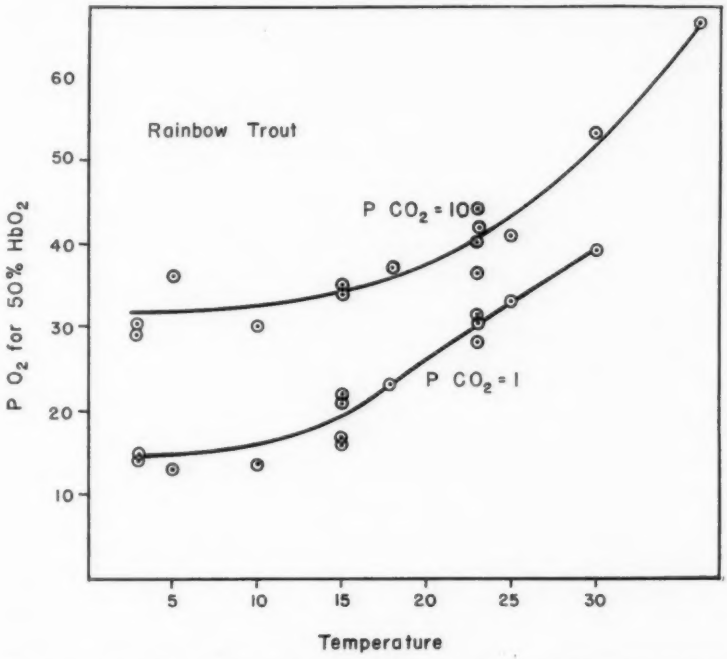

FIG. 5. The effect of temperature upon the pressure of oxygen necessary to half-saturate blood of rainbow trout at CO_2 tensions 1 and 10 mm.

tures, to estimate the effect of temperature upon oxygen affinity before the blood deteriorated.

The effect of temperature upon half saturation of hemoglobin in brown trout blood is shown in Fig. 3, the upper curve at 10 mm. CO_2 tension and the next lower at 1 mm. CO_2. For comparison with human blood the lower curve is drawn from the data of Brown and Hill (1923), and points for dog's blood at 37° (Bohr, Hasselbalch, and Krogh, 1904) show how CO_2 affects its affinity for oxygen. Data for the effect of

temperature upon blood of brook trout and rainbow trout are shown in Figs. 4 and 5.

When the curves for the three species are superimposed in Fig. 6, it is apparent that without CO_2 there is no difference between the blood of brook and brown trout, but the hemoglobin of rainbow trout requires somewhat greater pressure of oxygen to secure half saturation, particularly at lower temperatures. In the absence of CO_2, the bloods of the

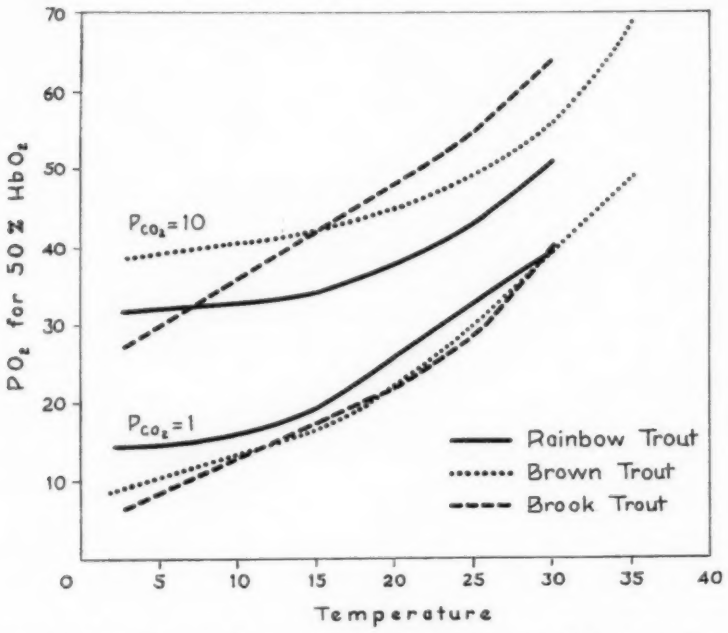

FIG. 6. Comparison of effects of temperature upon oxygen affinity in blood of brown, brook, and rainbow trout.

three trout are quite similar, but with 10 mm. CO_2 tension significant differences are apparent.

In the blood of several fish (Root, 1931; Willmer, 1934; Black, 1940), typical oxygen dissociation curves obtained in the absence of CO_2 are shifted to the right by CO_2. The shape of the curve may be altered as well as its position (Root, 1931), and one change in particular appears in the failure of the hemoglobin to become saturated with oxygen even at tensions of 150 mm. As a consequence of this situation the

tension of O_2 required for half saturation cannot serve as in the absence of CO_2 to define the whole dissociation curve.

Up to about 65 per cent saturation, however, oxygen dissociation curves in the presence of 10 mm. CO_2 rise approximately straight from the origin. If the oxygen tension for a single degree of saturation between 40 and 60 per cent is determined, that point may then be used to designate the curve as far as the 65 per cent level without appreciable error, and the tension at half saturation locates part of the curve as well as the important physiological condition during unloading of oxygen.

The effect of 10 mm. tension of CO_2 upon the oxygen tension needed for half saturation is shown by the upper curves in Figs. 3, 4, and 5 at each temperature. The distribution of points at any temperature amounted to about 10 mm. in the blood of rainbow and brown trout,

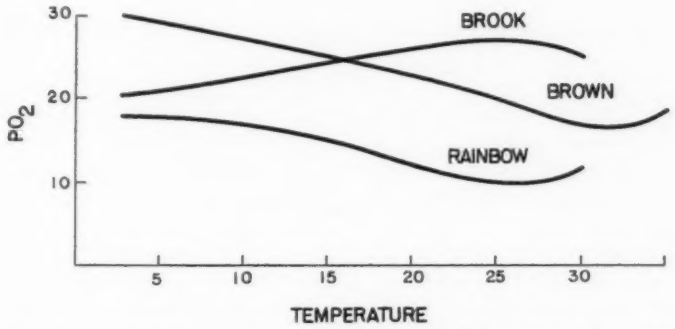

FIG. 7. Increase in PO_2 necessary for half-saturation when PCO_2 is increased from 1 to 10 mm. at different temperatures.

but was less in brook trout. The curves do not miss any point by more than 5 mm., and describe the condition of the blood closely, considering the number of samples of blood examined.

When the curves are superimposed in Fig. 6, it is apparent that at 15° the blood of brook and brown trout are alike with and without CO_2. The effect of CO_2 upon rainbow trout blood is less by nearly 10 mm., which is a greater difference than is shown by individual variations. Over the full range of temperature the curve representing brook trout blood with CO_2 differs from the curve for the other two species in position and slope.

If the addition of CO_2 to the blood occurred in the tissues, the diminished affinity of hemoglobin for oxygen would facilitate the diffusion of oxygen from the blood into the tissues. The situation resembles that

produced by the Bohr effect in favoring unloading of oxygen from human blood, but in the blood of many fish the influence of CO_2 is much greater. In Fig. 7 there is shown the influence which 10 mm. CO_2 would have in raising the oxygen tension at half saturation and at various temperatures. At 5° the addition of 10 mm. CO_2 requires a rise in oxygen tension for half saturation of 17 mm. in rainbow trout, 20 mm. in brook trout, and 30 mm. in brown trout. The oxygen tension is, however, three times increased by CO_2 in brook trout and only doubled in rainbow trout. The practical bearing of specific differences of this size upon the unloading tensions of oxygen would be important in respiratory transport. It is indicated that the blood of the three species suits its use under different conditions, particularly at temperatures away from 15°, but during unloading in the tissues rather than during loading in the gills.

Properties of the Erythrocytes Related to the CO_2 Effect

Effect of Hemolysis

It is an easy custom to attribute differences among different specific types of blood to specific properties of the hemoglobin involved. But the pure hemoglobin of fish is not known, and there are some striking illustrations that in fish blood the properties of hemoglobin depend upon its condition within the erythrocyte. The effects of CO_2 upon O_2 affinity largely disappear from the blood of the carp and sucker when the blood is hemolyzed (Black and Irving, 1938). The CO_2 effect likewise depends upon the integrity of the erythrocytes in the blood of the tautog and toadfish. On the other hand, the hemolyzed blood of the sea robin still retains part of its sensitivity to CO_2 (Root, Irving, and Black, 1939). The blood of the Atlantic salmon (Irving, 1939) is still sensitive to CO_2 after hemolysis, and so there are instances for the dependence of CO_2 sensitivity upon the erythrocytes in some species and independence in other species.

Samples of normal trout blood and of blood hemolyzed with saponin were equilibrated with 150 mm. pressure of oxygen and several pressures of CO_2 up to about 80 mm., according to the procedure outlined earlier for determining the limit of the CO_2 effect. The CO_2 effect in hemolyzed blood proved to be only slightly less than in whole blood. The limit of the CO_2 effect was about 50 per cent reduction of the hemoglobin in whole blood and about 40 per cent reduction in hemolyzed blood. Evidently the hemoglobin of trout blood, like that of salmon and sea robins, is sensitive to CO_2 whether in the erythrocytes or in hemolyzed blood.

Changes in Cell Volume

We have observed that the erythrocytes of a number of fish swell to a remarkable extent when the CO_2 tension is increased. The erythrocytes of suckers (Black, 1940), tautog, sea robins, and toadfish (unpublished observations), and Atlantic salmon (Irving, 1939), and rainbow trout (Ferguson and Black, 1940) swell considerably, while the cells of carp (Black, 1940) swell very little with CO_2. In the rather small number of species examined swelling is great in those in which the CO_2 effect is large, and small where the CO_2 effect is small.

Comparing the erythrocyte volume of the blood of the trout when the blood was equilibrated with air, with the erythrocyte volume when the same blood was equilibrated with 10 mm. CO_2 and about half saturated with oxygen showed that CO_2 caused swelling in every case. These volume changes are shown in Table III as the increase in per-

TABLE III

Increase in volume of erythrocytes produced by 10 mm. CO_2

Species	Number	Range of swelling	Average of swelling
		per cent	*per cent*
Brown trout *Trutta trutta*	14	1–21	10
Rainbow trout *Trutta iridea*	25	4–27	15
Brook trout *Salvelinus fontinalis*	10	1–24	9

centage over the original volume. Removal of the CO_2 reduced the volume of the erythrocytes again. The swelling of fish erythrocytes with CO_2 is more variable than would be expected if CO_2 tension is the only variable factor which determines volume changes, and erythrocyte volume is certainly labile toward factors or conditions other than CO_2 tension alone.

CO_2 Dissociation Curves

The data from the determinations of CO_2 effects were plotted logarithmically as content against tension of CO_2. In all of the blood samples considered the hemoglobin was about half saturated. The points were uniformly distributed, and the regularity justified the construction of the average curves shown in Fig. 8. The points show the mean position of the results obtained with each species, and indicate that there was no difference between the average blood of brook and brown trout.

Blood of rainbow trout has, however, consistently a somewhat smaller CO_2-combining power than the blood of the others. Judging from the slope of the curves, the buffering of the blood of the three species is similar at CO_2 tensions greater than 10 mm. Up to 10 mm. the buffering of brook and brown trout somewhat exceeds that of rainbow trout, although the concentration of hemoglobin in the latter is usually larger than in the other two species.

The amount of CO_2 combined with blood increases with diminishing

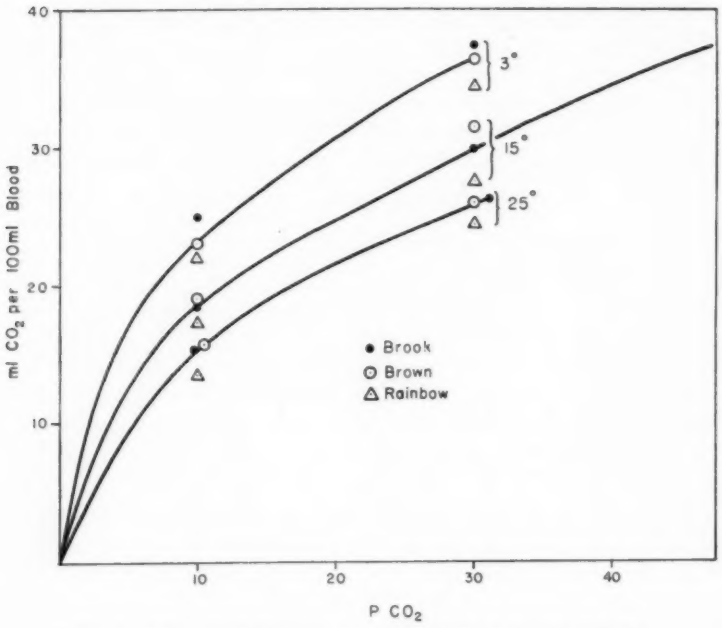

FIG. 8. CO_2 dissociation curves of trout blood at 3°, 15°, and 25°.

temperature more rapidly than does the solubility of CO_2 alone. If the CO_2 added to the blood increases the anion concentration, base must be removed by CO_2 from the protein buffers, which, as weak acids, must then undergo a decline in strength in comparison with carbonic acid. The amount of base lost by proteins and gained by CO_2 is equivalent to the change from 26.0 to 36.0 volumes per cent of combined CO_2 between 25° and 3° or 4.5 millimols per liter. Along with this decline in base-binding power of the protein, the affinity of the hemoglobin for oxygen increases as the temperature declines.

Increasing the temperature diminishes the affinity of the hemoglobin for oxygen and increases the ability of the hemoglobin to bind base. The affinity of hemoglobin for oxygen may also be diminished by acidification, but with an opposite effect upon ability to bind base. From these contrasting relations between the effect of temperature and acidity upon oxygen affinity it appears likely that change of temperature does not alter the affinity of hemoglobin for oxygen by affecting its ionization.

Discussion

The affinity for oxygen of the blood of the three species of trout is scarcely distinguishable at 15° and in the absence of CO_2. In the arterial blood of trout the tension of CO_2 is probably only one or two millimeters (Ferguson and Black, 1940), and complete oxygenation in efficient gills would be equally secured for all three species by atmospheric tensions of oxygen in water cooler than 25°. At 20° half of the atmospheric tension of oxygen would suffice to saturate the blood, and it seems likely that under common natural conditions of temperature and oxygen supply the blood of all three species would be sufficiently and about equally oxygenated.

But when the effects of CO_2 and changing temperature are considered, differences appear which distinguish each species. At 5° the normal tension of CO_2, which is about 10 mm. in venous blood, would yield, at half saturation, as unloading pressure, in brook trout 29 mm., in rainbow trout 32 mm., and in brown trout 39 mm. At 25°, the pressures would be 54 mm. in brook trout, 47 mm. in brown trout, and 39 in rainbow trout. The condition of half saturation and with 10 mm. tension of CO_2 represents the situation in the blood when O_2 is passing into the tissues. At low temperature the tension of O_2 available for unloading would be greatest in brown trout, while the blood of brook trout would offer greater unloading tension at higher temperature. The characteristics of unloading are distinct for each species at all temperatures except at 15°, and the change in unloading conditions with temperature is likewise distinct in the blood of each species. The natural consequence would afford to the brown trout an unloading tension greater than that of the other three at low temperatures. At high temperature the brook trout would have the advantage of greater unloading tension.

The specific differences which have been shown might be attributed to the possession of hemoglobin of a different type by each species, but on this point there is no evidence. Another view could regard the differences as based upon the influence of the milieu upon the oxygen affinity of a type of hemoglobin common to the three species. Hemoglobin is extremely sensitive to the acidity and salts in which it exists, and in

natural conditions the slope of the CO_2 curve in trout blood is very steep. The changes in acidity within the erythrocyte may be quite rapid, and that the osmotic changes are apparently quite large is indicated by the considerable swelling of erythrocytes produced by a 10 mm. increase of CO_2 tension. We have not seen any specific difference in the erythrocytes which would affect oxygen affinity, but the differences which we have shown are small, and our observations upon the lability of the erythrocytes are gross. The influence of temperature is undoubtedly exerted directly upon the hemoglobin, but temperature probably influences the properties of the erythrocytes as well, and so indirectly affects the hemoglobin by altering its milieu. The lability of the milieu and the sensitivity of hemoglobin are such that the respiratory functions of quite similar hemoglobins might be greatly modified by the milieu.

Summary

Various species of fish possess blood with different characteristic ability for combining with oxygen. These differences appear to fit the blood of each species for the transport of oxygen under special conditions.

In three closely related species of trout the characteristics of oxygen combination are similar at the low CO_2 tension characteristic of arterial blood. The effect of rising temperature upon the combination of oxygen with the blood *in vitro* of *Salvelinus fontinalis, Trutta trutta,* and *Trutta iridea* is to diminish the oxygen affinity. At 15° their blood is half saturated at 17, 17, and 18 mm. tension of oxygen respectively, and changing temperature increases the oxygen tension required for half saturation about 1 mm. per degree. This situation prevails when the tension of CO_2 is about 1 mm., and only at lower temperature does the blood of rainbow trout become distinguishable from the other two in requiring slightly greater oxygen tension for half saturation.

CO_2 greatly decreases the affinity of the hemoglobin for oxygen. The limit of the effect of CO_2 is reached at about 60 mm., and at that tension at 15° the hemoglobin is only half saturated. Raising the temperature diminishes the degree of oxygen saturation in the presence of CO_2.

When the CO_2 tension is 10 mm., half saturation with oxygen requires about twice the tension of oxygen needed in the absence of CO_2. The curves representing change in oxygen affinity with temperature when the CO_2 tension is 10 mm. are different in position or slope for each of the three species. The differences are large enough to fit the blood of each species for oxygen transport under different conditions.

The erythrocytes of trout blood may swell 25 per cent when the CO_2 tension is increased from one to 10 mm. The swelling is observed in the blood of several species of fish having hemoglobin which is sensitive to CO_2. The CO_2 dissociation curves of the three trout are essentially alike and vary in the same manner with temperature.

The difference observed in the blood of these three species would apparently provide different conditions for unloading oxygen in the tissues, and the change of unloading conditions with temperature is peculiar to each species of trout. Only at temperatures above 20° would aeration at the gills normally be restricted.

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ON THE METHOD OF FEEDING OF FOUR PELECYPODS

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Introduction

The feeding tracts of many pelecypods have been described, but while many of these accounts have approximated the true conditions, there is one important fact that has been omitted, and it is upon this that an understanding of their feeding methods depends.

When the pelecypods used in this experiment were feeding, a sheet of mucus entirely covered the gill structure, and it is this mucous sheet which strains out food material from the water vascular current. Cilia serve only to create the current and move the mucus. With the exception of highly specialized or quite primitive forms, it is probable that this method of feeding is general in the class Pelecypoda.

Except in cases where the animal has been left sufficiently long (usually long enough to begin the regeneration of the shell and mantle), a pelecypod which has been cut open is not feeding. When disturbed, the pelecypods that I have investigated cease feeding at once, and, when brought into the laboratory, several days may elapse before they will feed naturally. Therefore, it is necessary to make certain that the animal which is being investigated is adjusted to its surroundings and is carrying on its activities exactly as though it were in its natural habitat. Many pelecypods will begin feeding shortly after being brought into the laboratory, provided they are not mutilated in any way. In such cases feeding may begin within a few hours, and, in time, these animals actually become somewhat adapted to oft repeated disturbances.

Materials and Methods

Four pelecypods were used, namely, the gaper clam, *Schizothaerus nuttallii;* the mud flat scallop, *Pecten circularis;* the native West Coast oyster, *Ostrea lurida;* and the West Coast mussel, *Mytilus californianus.* These represent a burowing form, a surface form, an above surface form, and an open coast form. The first three use detritus for food, and the fourth uses plankton.

A hole was bored in different positions in one side of the shell of different individuals of each species, so that ultimately all regions connected with the feeding activities of each of these pelecypods could be observed. These windows were covered with a thin piece of glass, the size of the window depending upon what region and how much area of the region was to be investigated.

The openings were made by grinding a portion of the shell away, removing the underlying mantle, and then cementing a piece of cover-glass over the hole. The cover-glass was cut to shape by means of carborundum points and cemented in place with United Mender. Many cements were used, but the United Mender made by the United Sales Co. of New York, Dallas and Los Angeles proved to be by far the best; for windows cemented in place with it remained in place for as long as six months, although they were continuously immersed in ocean water. Before the cement was applied both the cover-glass and the rim of the shell around the opening were wiped clean with a clean cloth dampened with 95 per cent alcohol.

It is necessary to have the shell surrounding the opening perfectly flat before the cover-glass is cemented in place. A sander grinds faster and generates less heat than an emery wheel. While one is grinding the opening the animal should be dipped often into ocean water. A small hand rotor and dentist's drills can be used to advantage to cut the opening, and then only the surface surrounding the opening need be sanded flat.

A binocular and microscope lamp were suitably mounted next the aquarium in such a manner that they could be adjusted to any position.

After a window was put in a shell the animal was left undisturbed in running sea water for about two weeks, then watched carefully through the windows with the binocular to determine if feeding was being carried on in a natural manner. This was determined by introducing into the water some non-irritating material which constitutes the natural food of the animal.

The food material used was either a diatom culture or detritus, the latter being preferable. When the surface of the mud of an estuary or of the ocean is disturbed, a grayish turbidity results. The material causing this turbidity consists of decaying organic matter which is rich in bacteria, protozoa and other organisms such as rhabdocoeles, nematode worms, larvae of many species of marine animals, and, in addition, on mud flats there are usually surface diatoms and single-celled algae. This surface sediment or detritus, which constitutes the main, or, in many cases, the only source of food for burrowing or surface pelecypods, is

obtainable in any quantity and is readily eaten by most pelecypods. When introduced as food it does not disturb the feeding activities of the clams. (A diatom culture was found to be better for *Mytilus californianus*.)

Feeding

In the four species listed above, when feeding begins either mucus is secreted at the upper edges of the gills and is carried in a sheet by the frontal cilia to the free edges of the gills, or it is secreted more or less uniformly over the entire surface. It is then carried in strings along the edges of the gills to the labial palps. The palps perform a selective function, at least to the extent of partially removing undesirable particles, while allowing the rest of the material to pass intact with the strings of mucus directly into the esophagus in the form of food-laden strings of mucus. The main point to be stressed here is that the sheet of mucus covers the entire gill in much the same manner as described for tunicates (MacGinitie, 1939), and intercepts all particles from the current of water which passes through the gills and out through the dorsal or excurrent channels.

I consider a pelecypod to be feeding when a sheet of mucus entirely covers the gills, at which times all particles in the water, however small they may be, are strained out by the mucus. I am referring to undisturbed animals. Mucus may be made to flow copiously from any portion of a gill by direct stimulation, but it is very difficult to determine just where and when the secretion of mucus for feeding takes place. For this reason it took more than two years of careful observation to be sure of the main points set forth in this paper. Since mucus itself is perfectly transparent, the presence of the sheet of mucus is shown only by the included detritus. When feeding is actually going on, as witnessed through a window in the shell, the sheet of mucus may not follow the grooves, but, as shown by particles in it, may be deflected somewhat in an anterior direction, the particles carried by it crossing over the grooves, for they are carried by the mucus and not by the cilia. For example, when the mucus sheet is present in *Mytilus californianus*, the pull of the cilia of the free edge of the gills causes the sheet to be deflected ahead, particularly near the lower edge of the gills. Thus it is seen that the sheet of mucus, and not the cilia, carries the food particles.

When particles are moved by the cilia in the absence of the mucous sheet, as in the case of an opened clam when one valve and mantle have been removed, such particles follow the grooves. As it is the frontal cilia which move the mucus, it is to be expected that when the mucous sheet is absent, that is, when feeding is not taking place, the particles

being moved to the free edge of the gills will move parallel to the grooves. The current of water created by the lateral cilia bordering the grooves tends to hold the particles in the grooves as they are being moved by the frontal cilia towards the free edges of the gills. In some pelecypods particles will move both down the ridges and up the grooves. The ciliary mechanisms have been worked out in great detail by Atkins (1936, 1937, 1938).

When a small amount of carmine powder is mixed with the detritus and introduced with the incurrent water, it is usually ingested. In *Mytilus californianus* and *Schizothaerus nuttallii* if carmine alone is introduced in the same manner, some of it will find lodgment on the sheet of mucus already formed; but the sheet nearly always will be cut off at the upper edge and the carmine which thereafter collects on the gills will be carried down the grooves, thence forward along the edge of the gill, and then dropped by the labial palps into the anterior portion of the mantle cavity. In *Mytilus californianus* rejected material that is dropped into the anterior portion of the mantle cavity is carried posteriorly by grooves just within the mantle edge and issues from the posterior end in a continuous string termed pseudofeces. I have carefully observed, through windows at the anterior portion of several *Mytilus californianus*, the rejection of the undesirable material by the labial palps. The palps spread apart and assume a transverse position. The mucous threads from the edges of the gills travel directly to the bases of the ventral palps, down their anterior edges and thence into the ventral grooves of the mantle edge, where the laden threads of mucus travel to the posterior end and out as the pseudofeces mentioned above. When *Mytilus californianus* is feeding, the palps are laid backward outside of and close to the gills.

In *Schizothaerus nuttallii* rejected material will be forcibly ejected from the mantle cavity by a sharp contraction of the adductor muscles, which quickly brings the valves together and squirts the water and rejected material from the mantle cavity out through the incurrent siphon or opening.

This activity undoubtedly accounts for some of the squirting by clams on mud flats when the tide is going out. As the tide is leaving the mud flats, clams that have long siphons and burrow deeply squirt much more often than they do when the tide is in and they are covered by water. When the mud flats are nearly bare a considerable amount of sand and other undesirable material stirred up by the action of the waves may cause material that will be rejected to accumulate rather rapidly. Long-necked clams usually eject water much more forcibly than do those clams which live much nearer the surface. *Schizothaerus nuttallii*

squirts water to a height of from 3 to 5 feet. Such removal of rejected material, or squirting, is also much more frequent when the tide is first coming in, as will be evident to anyone who will take the trouble to stand knee deep in the incoming tide in a clam bed and observe.

The mantle cavities of the four pelecypods were never free of mucus, and particles are at all times conveyed to and along the edges of the gills by mucus, but it is only at feeding times that the gills are covered by the sheets of mucus. While feeding is going on these sheets are being continuously secreted and move slowly toward the free edges of the gills.

It should be noted here that some mucus with its included particles may find its way into the mouth when the sheet of mucus is not present. I think this is sometimes due to testing the mucus for suitable food, for the secretion of the mucous plate often follows such testing. Abnormal ingestion of material often follows disturbance or mutilation. This is clearly shown by placing carmine or carborundum on the gills of a pelecypod which has been opened. Under such conditions the introduced material may be carried to the mouth and ingested. This never happens in the case of a pelecypod which is feeding normally in an aquarium as observed through a window, for the introduction of even small amounts of carmine or carborundum causes the pelecypod to cease feeding immediately. I consider carborundum particles the most undesirable of all materials to use in feeding experiments.

In connection with the above, I am of the opinion that the function of the osphradia as "water testing organs" is over-emphasized in the pelecypods. The region which seems to me to have the highest tactile and "olfactory" sense is the region where the incurrent opening is located. In *Mytilus, Pecten* and *Ostrea* it is the edge of the mantle; in *Schizothaerus* it is the finger-like papillae which partially close the entrance of the incurrent siphon and act as a coarse strainer. When a valve has been removed, part of the reception area for stimuli has been removed, and the nervous system of the pelecypod is rather badly upset, to say the least. What an animal does with the mucous threads from its gills at such a time had better be disregarded.

Discussion

It is unwise to speak of feeding in a pelecypod unless it is actually observed doing so. Pelecypods are very sensitive to stimulation, either mechanical or chemical (Hopkins, 1932*a*, 1932*b*), and sometimes will cease feeding at the least movement or change in food material. In general, I think it may be said that small or juvenile members of any

species are less sensitive than the older and larger ones, for they adjust themselves more quickly to handling and begin feeding sooner after the window is placed in them. For this reason it is best to use as young specimens as one can conveniently. They are quite erratic for several days after being moved into the laboratory, and also after any major disturbance. However, when once fixed and left alone for a considerable length of time, which varies in each individual, they become much more uniform in their feeding activities, although apparently none of them ever feed continually. The rate of intake of water varies considerably, and this is usually or perhaps always accompanied by some contraction of the gills. Complete contraction of the gills shuts off the current of water entirely, just as a similar contraction does in the tunicate basket (MacGinitie, 1939). It is impossible for large particles to pass through the gills. Whether such small particles as bacteria pass through or not depends on whether or not the pelecypod is feeding.

It is well known that cilia often have a selective function, fine examples being the cilia of the pouch and funnel of *Stentor* (Schaeffer, 1910), and the egg and sperm collectors of *Urechis* (MacGinitie, 1935). Nevertheless, the separation of solid material from water currents is much more efficiently done by straining such water through mucus. It is not surprising, therefore, that mucus plays a much more important rôle in the feeding mechanisms of plankton and detritus feeders than it has been given credit for doing. Certainly it never should be said that a pelecypod is feeding just because it is pumping or maintaining a current through the mantle cavity.

In the light of the information presented here, it is interesting to read other papers concerned with the feeding of pelecypods and even certain gastropods (Orton, 1912). When the use of a mucous sheet for straining food material from the water is understood it will eliminate many points of discussion that have arisen. Although many papers concerned with the feeding of pelecypods have been written, only a few are listed in this paper, for the literature on this subject is rather voluminous. Therefore, the reader is referred to the following papers for complete bibliographies on the subject (Atkins, 1936–38; Galtsoff, 1928; Hopkins, 1936; Nelson, 1938; Orton, 1912; Yonge, 1936; and ZoBell and Feltham, 1938).

When it is understood that the food material of pelecypods in general is strained from the water as it passes through a sheet of mucus, and that feeding is being carried on only when such a sheet is present, it will clear up practically all points of uncertainty that one meets in reading about feeding methods and feeding experiments in the pelecypod mollusks.

SUMMARY

1. Openings were made through the valve and mantle of four species of pelecypods. These were made in various positions in the valves of many individuals so that ultimately all outer portions of the feeding mechanisms could be observed. These openings were covered with pieces of cover-glass cemented in place so as to form windows through which the feeding activities could be watched.

2. The feeding activities were observed through a binocular without in any way disturbing the animals.

3. Evidence is given to show that when a pelecypod is feeding a sheet of mucus covers the gills, and it is this mucus which strains the food material from the water, the cilia affording mechanical means for its transportation.

4. While the pelecypod is feeding this mucus is constantly being secreted and is carried to the food grooves bordering the gills, along which it is transported to the mouth as strings of food-laden mucus.

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THE COLORATION AND COLOR CHANGES OF THE GULF-WEED CRAB, PLANES MINUTUS

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Many observers have noted the remarkable adaptations in color pattern of the gulf-weed fauna. Perhaps the most common and colorful crustacean associated with the gulf-weed is the little grapsoid crab, *Planes minutus* (L.). Its predominant color is brown of many shades from yellow to red, matching the weed to which it clings. The brown of many individuals is broken by conspicuous white patches of various shapes and sizes, some sufficiently large to cover the entire carapace. These white areas appear to be in imitation of the conspicuous calcareous tubes of the annelid worms attached to the gulf-weed. A color plate showing a few of the variations is given in Murray and Hjort's book (1912). Little experimental work has been done with this crab. Crozier (1918), who found some mahogany-colored *Planes* on a drifting tree of that shade cast ashore at Bermuda, was unable to detect any change in coloration after they had been kept for six days on the much lighter gulf-weed. The coloration and color changes of one of the other members of the gulf-weed fauna, the shrimp, *Latreutes fucorum*, have recently been described by Brown (1939).

In an attempt to discover whether the coloration of *Planes* is a fixed pattern or an active adaptation to background, crabs were kept on different backgrounds and observed microscopically as well as grossly. A white background was furnished by white china bowls. Other backgrounds were obtained by painting the outer surface of clear glass dishes. Ordinary commercial paints were used for this, the green, for example, being of a dark shade marketed as " window blind green." The crabs were exposed to these backgrounds for one day in full sunlight from dawn to dusk. At sundown the bowls were placed under a bright electric light until the crabs could be examined. Microscopic observations were made on the flattened surface of the fifth leg, a region easily viewed under low magnification ($\times 62$). The results of these observations are summarized in Table I.

[1] This study was made possible by a grant from the James F. Porter Fund of Harvard University.

There are three kinds of chromatophores in *Planes minutus:* white, black and yellow. Of these the most prominent are the white cells, which usually appear to be larger and more numerous than the black cells. The yellow cells are smaller than either the whites or blacks, and are hard to distinguish except in the contracted state, both because of their diminutive size and because of masking by the other chromatophores. In some individuals these cells appear to be almost orange in

TABLE I

Responses of chromatophores in Planes minutus to different backgrounds

	O	*A*	*B*	*C*	Number of Crabs Examined
White Background					
Black cells	0	1	4	27	32
White cells	8	18	4	2	32
Yellow cells	3	2	12	15	32
Black Background					
Black cells	27	0	3	0	30
White cells	0	3	9	18	30
Yellow cells	18	6	6	0	30
Red Background					
Black cells	24	5	6	0	35
White cells	3	12	8	12	35
Yellow cells	10	4	15	6	35
Blue Background					
Black cells	0	0	5	18	23
White cells	13	9	1	0	23
Yellow cells	2	0	4	17	23
Yellow Background					
Black cells	0	1	4	21	26
White cells	12	13	1	0	26
Yellow cells	1	0	11	14	26
Green Background					
Black cells	1	8	5	5	19
White cells	5	5	5	4	19
Yellow cells	4	0	11	4	19

Symbols: O—pigment fully dispersed; processes indistinguishable.
A—pigment partially dispersed; arborizations visible.
B—pigment partially concentrated; stellate.
C—pigment fully concentrated; punctate.

Symbols after Kleinholz (1937). See his paper for illustrations of the four phases of pigment distribution.

color, and when viewed in the contracted state through the darker areas of the leg they appear to be ruby. It is probable that the yellow pigment is astacin, a carotinoid commonly found in Crustacea. This pigment appears red when concentrated. It may be that the ruby appearance is caused in part by the fact that they are viewed through a region of the exoskeleton which is dark-brown in color.

On most of the backgrounds tested each type of chromatophore re-

acted fairly consistently, its pigment tending to become either concentrated or dispersed. In general the black cells and the yellow cells reacted similarly, the only clear exception being when the animals were exposed to a red background. White cells and black cells reacted oppositely, the pigment of one becoming concentrated when that of the other became dispersed. The responses of the white cells to red and green backgrounds were not consistent, nor were those of the black cells to a green background. Perhaps examination of more animals or a longer exposure would have eliminated these apparent inconsistencies. However, it has been observed by others that there is in crustaceans some

TABLE II

Responses of Planes minutus compared with those of Portunus anceps and Portunus ordwayi

(*Data on Portunus from Abramowitz (1935)*).

Background	White Pigment Cells			Yellow Pigment Cells			Black Pigment Cells			Red Pigment Cells
	Planes minutus	*Portunus anceps*	*Portunus ordwayi*	*Planes minutus*	*Portunus anceps*	*Portunus ordwayi*	*Planes minutus*	*Portunus anceps*	*Portunus ordwayi*	*Portunus ordwayi*
White	D	D	D	C	C	C	C	I	C	C
Black	C	C	C	D	D	D	D	D	D	D
Blue	D	C	C	C	C	C	C	D	D	D
Red	C	C	C	C	D	D	D	D	D	D
Yellow	D	C	I	C	C	D	C	D	C	C
Green	—	D	C	C	C	C	—	D	D	I

Symbols: D—dispersed,
I—intermediate,
C—concentrated.

variability in chromatophoral response not only when different individuals are compared but also when different regions of the same animal are studied. The condition of the yellow pigment was frequently very difficult to determine, which introduced the likelihood of observational error.

In spite of chromatophoral responses, *Planes* is unable to effect color adaptation rapidly, for animals kept all day on white or yellow backgrounds became but slightly lighter than those kept on black. Almost every individual from one background can be matched in coloration in a group from a contrasting background. It is interesting to note that Abramowitz (1935) observed similar behaviour in two cancroid crabs. One of these, *Portunus anceps,* has the same three pigments as *Planes,*

while the other, *Portunus ordwayi,* has a fourth pigment, red. The red and black pigments were found to react similarly except perhaps to a green background, where the black became "dispersed" and the red "intermediate." Table II gives a comparison of the responses of *Planes* and the two species of *Portunus.* Since the responses of the cancroid crabs did not agree in all cases (one type of chromatophore apparently being concentrated in the one species and dispersed in the other for the same background), complete agreement between them and *Planes* is not found. However, if only those cases where the two species of *Portunus* are in agreement in their response are compared with *Planes,* there is lack of uniformity in only three instances: on a blue background for both the white and the red cells, and on a red background in the case of yellow cells. These differences are probably of no significance in view of the lack of consistent response which Abramowitz reported.

Direct comparison between the chromatophoral responses of *Planes* and *Latreutes* is difficult because the latter has red and blue pigments in addition to the white and yellow of *Planes,* and lacks black. The two species are alike in exhibiting a wide variety of colors and color patterns, but whereas *Planes* shows almost no alteration in appearance, *Latreutes* shows the effects of physiological color change almost immediately (Brown, personal communication).

The failure of *Planes* to effect a rapid color change in spite of its active chromatophoral responses may possibly be explained, at least in part, by a study of the moulted exoskeleton, which is a faintly colored replica of the intact skeleton. Each dark area or white spot on the intact animal is present in the exoskeleton, the intermediate areas being pale yellowish brown. Extra-chromatophoral pigment is found also in the hypodermal cells. Because of the distribution of this pigment, crabs whose chromatophores have reacted to a certain background do not become better color-adapted upon moulting. Until the diffuse pigment can be elaborated or destroyed, according to the prevailing condition of the chromatophore, it prevents the changed state of the chromatophores from becoming evident in the general appearance of the animal. This process apparently takes considerable time.

The pattern of the individual crab is probably genetic, as Brown has suggested for *Latreutes.* This is borne out by the observation made by Beebe (1928) that embryos of *Planes* have marked differences in pattern before hatching (p. 194). Yet it is clear from Crozier's report of the mahogany-colored individuals that *Planes* can in time become adapted to new backgrounds. The experiments reported above show that the chromatophores of *Planes* are responsive, but that extracellular

pigment in the hypodermis and exoskeleton prevents the animal from effecting an immediate change in appearance.

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SOME EXPERIMENTS ON THE EFFECTS OF HYPOPHYSECTOMY AND PITUITARY IMPLANTATIONS ON THE MALE FUNDULUS HETEROCLITUS [1, 2]

J. WENDELL BURGER

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While sufficient work has been accomplished to show that the hypophysis of fish secretes a gonadotropic principle, the hormonal relationships involved in the piscine sexual cyle are not clearly understood (compare Matthews, 1940 for a summary). For *Fundulus* Matthews (1939) found that after hypophysectomy, the testes failed to continue gametogenesis during the breeding period. The injection of mammalian pituitary extracts had no decisive effect upon the gonads. Matthews (1940) found, however, that the implantation of *Fundulus* pituitaries into non-hypophysectomized immature *Fundulus* induced gametogenic activity, especially in the male. The present investigations are complementary to those of Matthews. Positive effects from pituitary implantations were secured in hypophysectomized adult male *Fundulus*.

Effects of Hypophysectomy

On July 3–6, 73 freshly captured, mature male *Fundulus* were hypophysectomized. The opercular approach was used for this very simple operation. Control fish were given blank operations. All fish were maintained under identical conditions in running sea water, the temperature of which varied between 11° and 19° C. The water for the most part was near or below 15° C. The fish were fed almost daily on chopped clams. This diet appeared adequate, since the operated fish deposited fat as do fish in nature during the summer.

Mortality in operated fish was about 30 per cent. No significant difference was found between the mortality of hypophysectomized fish and those which received a blank operation. Over a two-month experimental period, the loss of the hypophysis seems to have little to do with the viability of *Fundulus*.

[1] Aided in part by a grant from the Penrose Fund of the American Philosophical Society; this grant administered in 1939–40 by T. H. Bissonnette.

[2] There is some doubt if this fish is strictly speaking, *Fundulus heteroclitus*. It may be a related heteroclitoid form.

At the time of hypophysectomy, the testes had passed their maximal development for the annual sexual cycle (Fig. 1). In nature, the maximal development occurring during the spring is followed by testicular regression. By late August, spermiogenetic transformations have almost ceased and the testes are practically devoid of sperm. As pointed out by Burger (1940), testicular regression does not occur as rapidly for fish kept in cool water (11°–17° C.) in the laboratory, as it does in the warmer water of the natural habitat. The degree of testicular regression attained by September 1, in fish which received blank operations, is shown in Fig. 2.

The effects on the testis after hypophysectomy were similar to those described by Matthews (1939) for other periods in the sexual cycle, viz., after complete testicular regression (October–December), and at the beginning of normal spermatogenesis (March–April).

The testes underwent a rapid reduction in size. The most obvious effect was a cessation of sperm formation. This cessation was not immediately a complete one. One month after hypophysectomy, however, only rare cysts of spermatids could be found. Two months after the operation, spermatids were absent in the six testes examined. At no time were spermatogonial multiplications suppressed. These divisions formed a well-defined cortical zone of spermatogonia. Cross-sections of testes from hypophysectomized fish are shown in Figs. 3 (August 2, one month after hypophysectomy), and 4 (September 1, two months after operation, cf. with control, Fig. 2). Thus it would appear that the loss of the hypophysis results in inhibition of spermatogenetic stages beyond those of spermatogonial division. Nevertheless,

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FIG. 1. Cross-section of a testis at time hypophysectomies were performed (July 3–6). This testis has passed its maximal sperm production. Black patches are sperm.

FIG. 2. Cross-section of a testis from a control fish, killed September 1; this fish has experienced a blank operation. Black patches are sperm. The cortical zone where spermatogenetic stages are visible, has become narrow (cf. Fig. 1).

FIG. 3. Cross-section of a testis from a hypophysectomized fish, killed August 2. The cortical zone contains almost nothing but spermatogonia. Spermiogenesis has practically ceased.

FIG. 4. Cross-section of a testis from a hypophysectomized fish, killed September 2. The cortical zone of spermatogonia has grown more narrow (cf. Fig. 3, one month earlier).

FIG. 5. Cross-section of a hypophysectomized fish implanted with twenty pituitaries, beginning August 17 and killed September 2. The spermatogenetic zone has deepened noticeably. Newly-formed sperm are visible. Compare with control, Fig. 4.

FIG. 6. Cross-section of a testis from a fish which received a blank operation; this fish was implanted with twenty pituitaries beginning August 17 and killed September 2. Compare with control, Fig. 2.

it does seem true that once spermatogenesis has been initiated, spermiogenesis can continue for some time, and to a limited degree, in the absence of the pituitary. The fact that this spermatogenesis is not maintained in any great volume even for as long as one month, indicates,

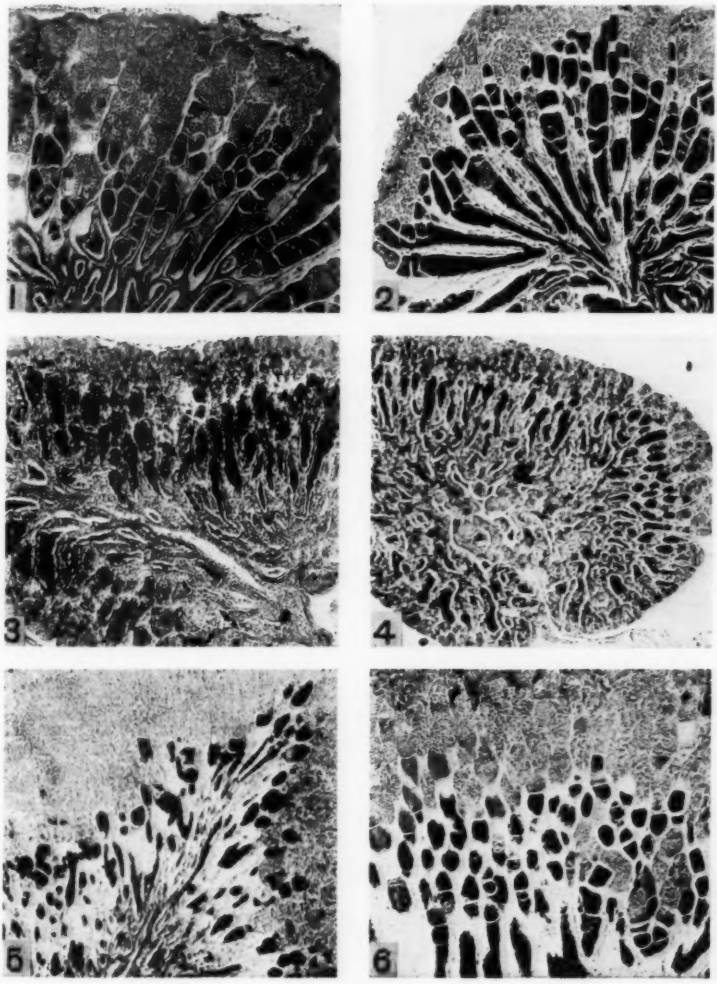

PLATE I

as Matthews (1939) has suggested, that the later stages of spermatogenesis are more sensitive to the absence of the pituitary than are the stages of spermatogonial division.

Effects of Pituitary Implantations

After the testes of the hypophysectomized fish had involuted sufficiently, both hypophysectomized fish and some operated controls were implanted with pituitaries from freshly captured, mature male *Fundulus*. The receptors selected were close to each other in size. The size of the donor fish was also roughly standardized. The implantations were made intraperitoneally. Ten hypophysectomized fish and three operated controls each received five fresh glands on each of the following days: August 17, 21, 26, and 29. These fish, which each received twenty glands, were sacrificed on September 2. Five hypophysectomized fish and two operated controls each received five fresh glands on each of the following days: August 21, 26, 29. These fish were killed on September 4. During this phase of the work, the water was almost constantly near 13° C.

The effects of the implantations were striking. Within five days after the first injections, the fish began to show pronounced sexual display antics. These peculiar swimming movements are common in fish during the breeding season and occur occasionally throughout the year in fish kept in aquaria. These movements were noticeably absent in the untreated hypophysectomized fish. By the tenth day after the first injection, the fish were in a frenzy of display.

Both series of implantations caused a recrudescence of the testes. After two weeks, the average weight of the testes of the fish which received twenty implants was slightly more than doubled, and the average volume was quadrupled, when compared with the average weight and volume of untreated hypophysectomized controls. The cortical zone of spermatogonia had deepened, while new transformations into spermatozoa were well established (Fig. 5). The hypophysectomized fish which each received fifteen glands likewise, formed new sperms. The weight and volume increases were the same as those for the fish which received twenty glands. The spermatogenetic stages were also the same. The non-hypophysectomized fish which were receptors of implants reacted as did the hypophysectomized receptors (Fig. 6). The control hypophysectomized fish (Fig. 4), and the control fish which experienced blank operations (Fig. 2) showed no testicular recrudescence during the experimental period.

These results clearly demonstrate that the pituitaries of adult male

Fundulus contain, and the testes of hypophysectomized and normal adult fish are responsive to, gonadotropic material. Matthews (1940) has shown that the immature testis of non-hypophysectomized *Fundulus* can be excited by Fundulus pituitaries implanted into the body cavity.

Discussion

The present study, together with that of Matthews (1940), permits an interpretation of the pituitary rhythm involved in the normal sexual cycle. During the period of sexual regression, pituitary secretion gradually declines. The pituitary does not abruptly cease to secrete, since active spermiogenesis in a gradually decreasing volume occurs during this regression. When the pituitary is removed, spermatogenesis is more quickly checked. In the late summer, pituitary secretion seems to be almost or entirely absent. Beginning in the fall and going through the winter, spermatogonial multiplications take place. The divisions can occur in the absence of the pituitary, but in the hypophysectomized fish there is no progressive increase in the number of these spermatogonia. Hence, the piling up in the testis of spermatogonia must be supported by hypophyseal secretion. The amount of this secretion is low, since very few spermatozoa are formed during the fall and winter. The spring spermatogenesis is accompanied by the highest phase of secretion. The gonadotropic material of the *Fundulus* pituitary is thus responsible for two phases of spermatogenesis: (1) the great volume of spermatogonial proliferation, and (2) maturation phenomena. It has been shown by Burger (1939) and Matthews (1939*a*) that the progressive phases at least of the sexual cycle of the male are influenced by the temperature of the water.

Summary

Adult male *Fundulus*, hypophysectomized shortly after maximal testicular development, show an inhibition of spermatogenesis for stages beyond those of spermatogonial multiplication. Spermatogonial divisions do not become numerous. The inhibition of the later stages is not immediately affected, since a few cysts continue to form sperm for as long as one month after hypophysectomy. These results confirm those of Matthews.

Both hypophysectomized adult male *Fundulus* and fish which received blank operations were maintained until sexual regression was well established. Implantations of twenty or fifteen pituitaries from normal male *Fundulus* caused within two weeks a recrudescence of the testes. Non-implanted controls showed none of this activity. It is concluded

that the pituitary of the adult male *Fundulus* contains gonadotropic material and that the testes of adult *Fundulus*, hypophysectomized or not, are responsive to this material.

The normal relation of the pituitary to the annual sexual cycle is discussed.

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MELANOSIS IN THE COMMON COD, GADUS CALLARIAS L., ASSOCIATED WITH TREMATODE INFECTION [1]

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A codfish displaying an unusual degree of melanosis is described in this paper. The fish was caught one mile north of Race Point, Provincetown, in March, 1940, by Mr. J. W. Lowes, who sent it to the Museum of Comparative Zoölogy. Mr. William C. Schroeder asked the writer to make a histological study of the tegumentary system of the specimen in a search for a possible clue to the cause of its melanosis.

METHOD

Samples of skin of one-half to one centimeter square were taken from different parts of the head, trunk and fins. The regions employed are indicated by letters and numbers shown in Fig. 1. Each sample was dehydrated, cleared and mounted in balsam. With a calibrated ocular micrometer ruled into squares, 3 to 5 separate square millimeters from

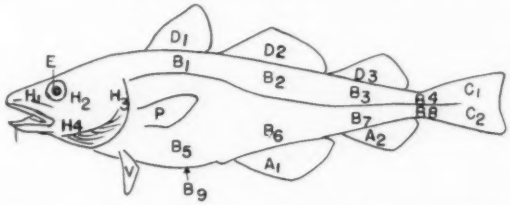

FIG. 1. Diagram of left side of cod showing regions from which skin samples are removed for comparative study. *A*, anal fin; *B*, body or trunk; *C*, caudal fin; *D*, dorsal fin; *E*, eye; *H*, head; *P*, pectoral fin; and *V*, ventral fin.

each piece of skin were measured under a binocular microscope, using reflected light, and the number of pigment cells per square millimeter determined and recorded from each region.

Small pieces of skin from the first dorsal fin (region *D 1*), the trunk, directly ventral to the first dorsal fin (region *B 1*), and the cornea (re-

[1] Contribution No. 253 of Woods Hole Oceanographic Institution.

[2] China Foundation Research Fellow.

gion *E*) were sectioned and stained with Heidenhain's "azan" stain which gives a blue color to the connective tissue, reddish yellow to the muscles, carmine to the cyst wall formed by the host tissue, blue to the cyst wall secreted by parasites and blue and carmine to the parasites themselves.

For comparison a normal cod was treated in the same way.

The trunk muscles, gills, oesophagus, heart and peritoneum were examined for parasites. As the fish had been eviscerated, only remnants of the cardiac portion of the stomach and liver were examined. The cysts were isolated, stained, and mounted and some of them sectioned and stained.

Observations

Superficial examination of the whole fish and microscopic study of sections of its skin show that general cutaneous melanosis in this fish is associated with parasitic infection which attacks the whole tegumentary

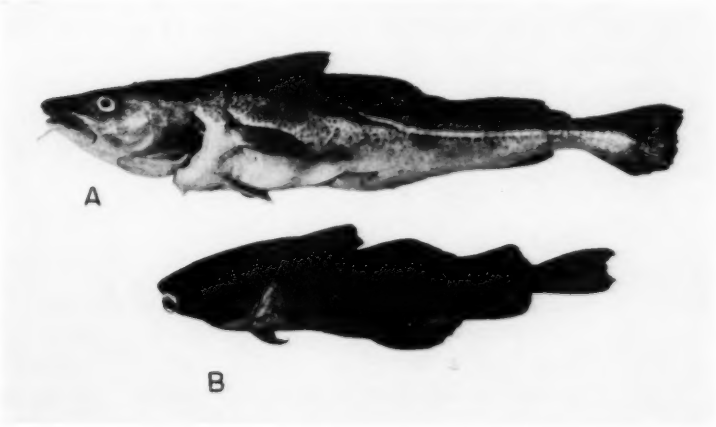

Fig. 2. *A*. Normal cod. *B*. Dark cod described in this paper.

system and the gill filaments. No parasitic cysts were found in the somatic muscles, the peritoneum, the heart, the oesophagus, the remnants of the liver and the cardiac end of the stomach which happened to be left in the fish after its evisceration. These uninfected regions exhibit no melanosis when compared with the corresponding regions in the normal cod.

In general appearance this fish is strikingly different from an ordinary cod in the presence of so many melanophores in the corneae and in the skin over the fins and the dorsal half of the body that these parts are actually black. The contrast in color between this and normal cod is shown in *A* and *B* of Fig. 2, which are printed from one photographic negative and hence are of identical exposure. Instead of being smooth and shiny, the skin is warty and rough. The tiny excrescences which produce the roughness are covered with more melanophores than the surrounding tissues. Parasitic cysts appear as white specks among the melanophores. Inside the cysts different stages of the metacercaria of a heterophyid trematode are seen.

The cysts and melanophores are so abundant in the cornea that the fish is blind and the eye scarcely distinguishable from the rest of the head. The melanophores on the body above the lateral line and on the dorsal and caudal fins are so numerous that they form a continuous sheet, making it impossible to ascertain their number per unit area of skin. In the less densely pigmented regions the dark cod has, on the whole, about six times as many melanophores per square millimeter on the head and six to nine times as many on the paired and anal fins as has a normal cod. Table I shows the number of pigment cells per square mm. for each of the 23 corresponding samples of skin from the dark and normal cod. The last two columns show that the melanophores of the dark cod are smaller than those of the normal fish. In the dark fish the melanophores are more uniform in size. In Fig. 3, *A* and *B*, two equal pieces of skin from the pectoral fin of a normal cod and this dark cod are compared. The normal cod has only one-sixth as many pigment cells as the dark individual.

Parasitic cysts are present in the tegumentary system from the tip of the snout to the surface of the caudal fin, including both corneae. When examined under a dissection microscope, they appear as small white dots among thick masses of melanophores—the tips of the cysts being free from pigment cells. From Fig. 3, *C*, it will be seen that the rugose appearance of the skin is produced by a mass of parasitic cysts under the epidermis which is thrown into folds. These cysts occur both above and below each scale, which, when pulled off from the body, always has a mass of cysts firmly attached to its two surfaces. The connective tissues are hypertrophied so that the skin is more than three times as thick as the normal skin from a corresponding part of the body (Fig. 3, *D*). The cornea is also infested. In Fig. 3, *F*, which is a photomicrograph of a 4 μ thick section of a piece of cornea, 10 trematode cysts can be seen from a field 1.86 mm. long and 0.66 mm. wide. This

cornea is more than twice as thick as a normal one and has melanophores throughout its whole thickness.

TABLE I

Comparison of melanophores between dark and normal cod

Body region	Number of melanophores		Ratio of melanophores: Dark / Normal	Size of melanophores in mm.	
	Normal	Dark		Normal	Dark
Head:					
H 1	25	121	5	0.15–0.2	0.1
H 2	10	72	7	0.3	0.1–0.2
H 3	12	72	6	0.3	0.15
H 4	9	63	7	0.3	0.25
Trunk:					
B 1	46	Too		0.2	0.05–0.1
B 2	51	numerous		0.2	0.05–0.1
B 3	55			0.2	0.05–0.1
B 4	82			0.1	0.1–0.15
B 5	7			0.3–0.4	0.1–0.15
B 6	7			0.3	0.15–0.2
B 7	17			0.2	0.2–0.25
B 8	14			0.4	0.15–0.2
B 9	1	19	19	Contracted	0.2–0.3
Dorsal fin:					
D 1	48	Too		0.15–0.2	0.1
D 2	39	numerous		0.2	0.1
D 3	55			0.15–0.2	0.1–0.2
Anal fin:					
A 1	5	40	8	Contracted	0.2–0.3
A 2	5	51	10	0.2–0.4	0.2
Caudal fin:					
C 1	87	Too		0.1	0.1
C 2	45	numerous		0.15–0.2	0.1
Pectoral fin:					
P 1	5	46	9	0.4	0.1–0.15
Ventral fin:					
V 1	6	37	6	0.2–0.25	0.2
Eye:					
E 1	0	66		0	0.2

The parasitic cysts are thick-walled, ovoid in outline and white to the naked eye. The majority of the cysts measure 0.33–0.38 mm. along one principal diameter and 0.24–0.28 mm. along the other. The cap-

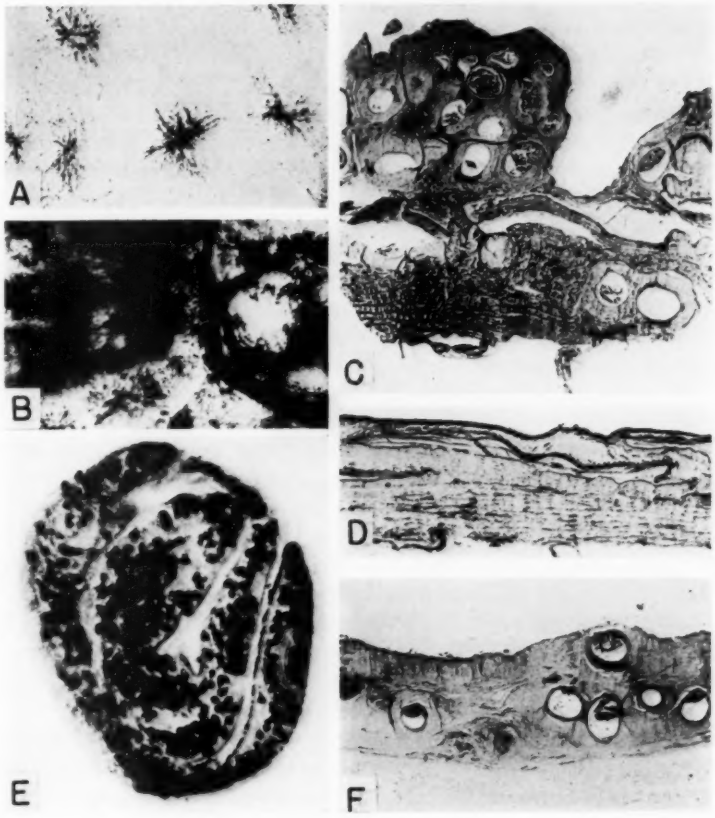

FIG. 3. Photomicrographs of preparations from cod skin.

A. Skin from the pectoral fin (same region as in *B*) of a normal cod.

B. Skin from pectoral region (*P* 1) unstained, showing part of the outline of two cysts and melanophores.

C. Section of a piece of melanotic cod skin from region *B* 1 (below the first dorsal and above the lateral line), showing the wavy epidermis and clusters of cysts. Section of one scale is shown in this figure. Three cysts are seen under the scale, while above it there is a large number of cysts.

D. Skin of normal cod from the same region and under the same magnification as in *C* above, showing one scale in section and part of two others and the smoother epidermis and less connective tissue.

E. Section of a metacercaria.

F. Section of the cornea showing cysts and pigmentation.

sules secreted by the parasites to enclose themselves measure 0.21 mm. by 0.14 mm. in the two principal diameters. The cyst walls are very resistant to mechanical injuries and are transparent in unstained skin cleared in xylene and mounted *in toto* in balsam. The cyst wall secreted by the host stains bright carmine with Heidenhain's "azan" stain and is laid down in concentric layers. It measures 0.028–0.07 mm. in thickness. The cyst wall secreted by the parasite, on the other hand, is only about 0.007 mm. thick and stains blue. In the gill filaments many smaller cysts are seen. This difference in size is due to the thinness of the host wall, for the smaller cysts contain parasites which, when measured along the parasite wall, are of the same size as those enclosed in the larger cysts of the gill filaments or the skin of the trunk. The host wall about the parasite increases in thickness, with more concentric layers, as the external size of the cyst increases. There is comparatively very little pigmentation about the cysts in the gill. All the melanophores present in the gill are arranged about the cysts with thick host walls. As the host walls are laid down about the parasites in concentric layers centrifugally and as the melanophores are associated with the more peripheral layers, it is probable that these pigment cells appear some time after the infection occurs.

The cyst contains the coiled body of the metacercaria of a trematode whose suckers can be distinguished through the transparent cyst walls. In stained sections (Fig. 3, *E*) the suckers and the spines on the posterior part of the body wall of the parasite can be seen easily. But as the metacercariae are still young, it is not possible to work out the structures of the reproductive system of our material.

From the absence of the parasitic cysts inside the body of the fish except in the tegumentary system and the gills, it may be inferred that the trematode larvae infected the cod by boring from the outside. The presence of very thin-walled parasitic cysts in the gill filaments indicates that these were the latest site of infection.

Compared with other infected fishes, this cod shows an extraordinarily heavy infection. Not only is the whole tegumentary system completely infested with parasites, but the parasitic cysts are gathered in groups several layers thick under the epidermis. According to Dr. Stunkard (verbal communication), a cunner kept in a laboratory aquarium for six weeks with 50 infected snails giving off thousands of trematode larvae does not get nearly so heavily infected. It is the more surprising when we consider that although in the ocean the cod was able to move about, it nevertheless contracted such an enormous number of parasites.

Discussion

Many cases of melanosis associated with parasitism have been observed among freshwater fishes. There are also a few records of melanosis in marine fishes parasitized by trematode larvae. In 1884 Ryder reported his observations on the darkening of the skin in parasitized cunners from Woods Hole and Cape Breton, N. S. He thought that these cysts were formed by the cercariae of some trematode and that the pigment cells about the site of infection were either formed *de novo* or gathered there by migration. Linton (1900) observed parasitic cysts on the skin of cunners in 1889, and in 1901 he reported similar infections on tautog, winter flounder, tom cod and eel and "less so on others." In 1915 he recognized the similarity between these encysted forms and the trematode *Tocotrema lingua* (Creplin). The presence of this species on the gills of sea raven and on the skin of cunners from Passamaquoddy Bay was reported by Cooper (1915). Stunkard worked out experimentally (1930) the life history of this trematode and identified it as *Cryptocotyle lingua* (Creplin), belonging to the family Heterophyidae—*Tocotrema* being long suppressed as a synonym of *Cryptocotyle*. Smith (1935) described a hyperplastic epidermal disease in two winter flounders associated with a trematode infection which was probably due to *Cryptocotyle lingua*. All these observations were probably concerned with the same species. A second species was reported by Gamble and Drew (1911) from Plymouth. A whiting infected by trematode larvae showed abnormal pigmentation in the form of black specks scattered over its pigmented areas and over the conjunctiva. They suggested that the trematode was a species of *Holostomum*, probably *H. cuticola*. No melanosis due to parasitism in the cod has been reported. In the cod described here the heaviness of infection and the intensive reaction of the host in the hypertrophy of the skin and the development of excessive melanophores are very remarkable.

Smith's experiments (1931, 1932) on the evoking of melanophores through mechanical injury and the eruption of corial melanophores and general cutaneous melanosis strongly suggest that these reactions are related to repair and defense of the tissues. The general eruption of melanophores in this cod, whose tegumentary system was completely infected by trematode metacercariae, is probably a defensive reaction against the parasite. There are different views on the question whether the melanophores in a parasitized fish migrate to the site of infection or are formed *de novo*. In this particular cod the number of melanophores is so much in excess of that found in an ordinary cod and they

so completely cover the whole body that migration of melanophores cannot account for them. They must have developed anew.

The development of the metacercariae is not advanced enough to allow an exact identification of the parasite. However, the mode of reaction of the host and the structures of the parasite, as far as they can be determined, appear very similar to what Linton described for *Cryptocotyle lingua*, which is found to infect a variety of fishes such as cunner, tautog, tom cod, eel, sea raven, winter flounder, etc. They suggest that this cod is parasitized by a species related to *Cryptocotyle*.

Summary

A very melanotic codfish is described which proved to be heavily infested with metacercariae of a heterophyid trematode. The number of parasites and the intensive reaction of the host in the development of melanophores and hypertrophy of the dermis are greater than any recorded for parasitized fish. The parasite may be a form related to *Cryptocotyle*.

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REPAYMENT OF THE ANAEROBIC OXYGEN DEBT IN GRASSHOPPER SKELETAL MUSCLE

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It has been shown (Gilmour, 1940) that in the roach *Cryptocercus punctulatus* Scudder, the "oxygen debt" incurred during a period of anaerobiosis is repaid threefold. Since such a large excess oxygen consumption during recovery had not previously been demonstrated in animal tissues, it seemed that further work on the phenomenon of oxygen debt repayment in insects was warranted.

In order to simplify the problem, it was decided to limit this investigation, as far as was possible, to one tissue. To this end, the hind femora of grasshoppers were employed as material. The femur of the jumping leg of the grasshopper consists almost entirely of skeletal muscle; the amount of epidermal and other tissue present would account for only a very small fraction of the total respiration.

Material and Methods

Two species of grasshopper were used: (1) *Melanoplus femur-rubrum* (De Geer). Adults of this species were collected in the field during the fall of 1939 and kept in the laboratory until used. (2) *Melanoplus differentialis* (Thomas). A number of adults were raised from eggs supplied by Dr. J. H. Bodine from the stocks at Iowa State University.

The legs were removed from the bodies by cutting through the trochanter. The tibiae were cut off close to the proximal end.

Oxygen uptake was studied in a differential volumeter designed especially for following oxygen debt repayment, the "apparatus B" previously employed by Rotta and Stannard (1939). The electrical conductivity method of Fenn (1928) was used in following carbon dioxide production. Oxygen uptake and carbon dioxide production of the resting leg were first followed in air, after which the vessels were filled with nitrogen, by running through the pure gas for 20 minutes,

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and the carbon dioxide production during anaerobiosis was determined; finally air or oxygen was re-admitted and the recovery respiration followed until a steady rate of oxygen uptake had been reestablished.

It was not possible to use the respirometer designed for conductivity determination for following both oxygen uptake and carbon dioxide production during the recovery period, as the oxygen uptake readings were disturbed by solubility effects. The anaerobic period was ended by passing pure oxygen through the vessel for one minute, insufficient time for equilibrium to be established between the gas mixture and the relatively large volume of barium hydroxide required by the method. Oxygen uptake was followed, however, at the beginning and end of each run with the conductivity apparatus, in order to establish the respiratory quotient.

TABLE I

Repayment of oxygen debt in isolated hind femora of *Melanoplus femur-rubrum*. The duration of anaerobiosis sometimes varied slightly from that shown in column 2. Such variations have been considered in calculating the amount of oxygen missed.

Experiment	Time in N_2	Initial O_2 uptake	O_2 missed	Final O_2 uptake	Excess O_2 consumed	$\frac{\text{Excess } O_2 \text{ consumed}}{O_2 \text{ missed}} \times 100$
	minutes	*mm.³/gm./hr.*	*mm.³/gm.*	*mm.³/gm./hr.*	*mm.³/gm.*	
G3	30	218	110	180	193	175
G5	30	180	90	173	160	180
G6	30	186	99	179	160	160
G9	30	269	135	277	250	185
G12	30	191	96	180	262	275
G4	60	158	158	162	173	110
G8	60	140	140	166	196	140
G10	60	201	201	162	550	275
Average		193		185		190

The temperature of the experiments was 23° C.

That an adequate supply of oxygen to the interior of the excised legs was maintained by diffusion from air was demonstrated by the fact that filling the vessels with oxygen caused no increase in oxygen consumption above that measured in air. The legs survived without any apparent disturbance in respiratory metabolism throughout the course of the experiments (5 to 9 hours). The oxygen uptake usually remained constant over long periods of time, although the final steady rate was often slightly lower than the initial.

Experimental Results

M. femur-rubrum

Either two or three legs were used in each experiment. The average oxygen uptake was 180 cu.mm. per gram live weight per hour (26 determinations ranging between 140 and 277 cu.mm. per gram per hour). The results of the oxygen debt experiments are shown in Table I. "Oxygen missed," in this table, means the amount of oxygen the tissue would have consumed in air, during the time it was in nitrogen. The last column represents the percentage repayment of the oxygen debt.

Table II

Repayment of oxygen debt and retention of carbon dioxide in isolated hind femora of Melanoplus differentialis.

Experiment	Initial O_2 uptake	RQ	Final O_2 uptake	RQ	Percentage repayment of O_2 debt	CO_2 produced in N_2	Expected excess CO_2 production	Measured excess CO_2 production	CO_2 retained
	mm.³/gm./hr.		*mm.³/gm./hr.*			*mm.³/gm.*	*mm.³/gm.*	*mm.³/gm.*	*mm.³/gm.*
M1	178	—	164	—	120	—	—	—	—
M2	180	0.93	180	0.80	—	136	225	212	13
M5	132	—	134	—	175	—	—	—	—
M6	142	0.79	132	0.85	—	126	178	174	4
M7	198	—	186	—	115	—	—	—	—
M8	212	0.88	216	0.79	—	154	265	20	245
M10	205	0.94	183	0.85	—	116	256	88	168
M13	207	—	195	—	160	—	—	—	—
M14	243	0.67	234	0.67	—	100	304	166	138
M15	253	—	212	—	80	—	—	—	—
M16	277	0.72	237	0.73	—	87	346	194	152
M17	202	—	201	—	110	—	—	—	—
M18	187	0.98	207	0.89	—	123	234	176	58
Average	201	0.84	191	0.80	125	120			111

There is no significant difference between the value for this obtained from the half-hour experiments and that from the one-hour experiments.

M. differentialis

One leg was used in each experiment. The procedure was to use the femur of one side of the grasshopper in the oxygen debt apparatus, and that of the other side in the respirometer designed for conductivity determination.

The average oxygen uptake was 197 cu.mm. per gram per hour (29 determinations ranging between 132 and 277 cu.mm. per gram per hour). Table II shows the results for both oxygen uptake and carbon dioxide production. The percentage repayment of the oxygen debt is calculated in the same manner as in Table I. The duration of anaerobio-

sis was one hour in all experiments. The "expected excess carbon dioxide production" is the amount of carbon dioxide which would have been given off, over and above that produced as the result of basal metabolism, if the recovery process had had a respiratory quotient of 1.0, and there had been no retention. The carbon dioxide retained in the tissues during recovery is the difference between this figure and the measured excess carbon dioxide production. The figures obtained in this way show an extremely wide range of variation, but have an average which is approximately equal to the average amount of carbon dioxide produced during anaerobiosis. The variation must be due largely to the fact that oxygen uptake and carbon dioxide production during recovery were not determined on the same tissue. In determining the expected excess carbon dioxide production it was supposed that the repayment of oxygen debt had the average value (125 per cent) in each case; that is, that the figure for excess oxygen consumed (and hence for carbon dioxide produced) was 125 per cent of the original oxygen uptake per hour. It might be supposed that a more accurate method would be to use, in each of the carbon dioxide experiments, the figure for oxygen debt repayment obtained from the opposite leg of the same grasshopper. When this is done, however, the variation is as great, while the average is practically unchanged (106 cu.mm. per gram). It appears, then, that it is impossible to predict the actual percentage repayment of oxygen debt of any leg, even from an experiment run on another leg of the same grasshopper. The average is thus the only figure for carbon dioxide retention that need be considered.

The respiratory quotient is somewhat low for muscle, but at the time at which experiments M14 and M16 were run the insects were rather inadequately fed, and the low respiratory quotients in these experiments are probably the result of the utilization of reserve foodstuffs.

Discussion

Since the chemical constituents of insect muscle are quite similar to those of vertebrate muscle, it is not unreasonable to expect the anaerobic processes of the two groups to be at least qualitatively similar. The fact that in *M. differentialis* the amount of carbon dioxide produced during anaerobiosis is equal to the amount retained during recovery suggests that the amount produced in anaerobiosis is the result simply of the buffering of acid by bicarbonate, and supports the conclusion that lactic acid is the only important end-product of anaerobiosis. In frog muscle 70 per cent of the debt incurred during anaerobiosis is repaid (Rotta and Stannard, 1939). In the insects used in this study more than 100

per cent is repaid. The problem of the removal of lactic acid thus seems to be a more expensive one in grasshopper muscle than in frog muscle, particularly in the case of *M. femur-rubrum*, which uses more oxygen in the recovery process than does *M. differentialis*. It has already been suggested that in *Cryptocercus* (*loc. cit.*) the threefold repayment of the oxygen debt may have been due to the burning off of a large proportion of the lactic acid supposed to have been produced by anaerobiosis. The fact that in isolated muscle tissue, investigated at normal temperature (as *Cryptocercus* was not), a repayment of the oxygen debt in excess of 100 per cent can be demonstrated lends support to this conclusion.

Summary

The average oxygen consumption of isolated hind femora of *Melanoplus femur-rubrum* was 180 cu.mm. per gram per hour; that of femora of *M. differentialis* was 197 cu. mm. per gram per hour. The average respiratory quotient of the latter was 0.82.

In *M. femur-rubrum* 190 per cent of the oxygen debt incurred during anaerobiosis was repaid during recovery. In *M. differentialis* 125 per cent of the debt was repaid, and the carbon dioxide retained in the tissues during the recovery period was equal to the carbon dioxide produced during anaerobiosis.

The end-products of anaerobiosis in grasshoppers are probably similar to those in vertebrates, but their removal seems to involve a greater expenditure of energy.

My thanks are due to Dr. W. O. Fenn for making available the facilities of his laboratory, and for his interest in the course of this work.

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GROWTH STUDIES ON CILIATES

VII. Comparative Growth Characteristics of Four Species of Sterile Ciliates

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During the past year experiments have been conducted on four species of holotrichous ciliates in pure culture in order to establish their nutritional requirements and some of their characteristics of growth. It is now possible to report the results of these experiments and to attempt an analysis of some of the factors of growth, both favorable and unfavorable.

In the ever widening field of protozoan physiology the quest is going on for more species which can be used for precise experiments. These species should be able to grow and reproduce in the absence of other microorganisms if complete control is to be obtained. Up to the present time it seems likely that the only genus of ciliate which has remained in successful pure culture is *Tetrahymena* (Furgason, 1940). Various names have been applied to pure-culture ciliates by different authors but, as Furgason has succeeded in showing, they were probably dealing with strains of *Tetrahymena geleii*. In a previous paper of this series (Kidder, Lilly and Claff, 1940) a description was given of a saprozoic ciliate which was referred to the genus *Glaucoma*. This organism (*G. vorax*) was described before access was had to Furgason's excellent work. I am now of the opinion that our ciliate belongs to the genus *Tetrahymena* and therefore it will be referred to in the future as *Tetrahymena vorax*.

Paramecium bursaria was cultured bacteria-free by Loefer (1936) but these cultures were subsequently lost. Because of the inclusions of *Chlorella* in this species the status of "pure culture" is questionable.

The four species to be dealt with in the following report are *Tetrahymena geleii* (strain W), *T. vorax*, *Glaucoma scintillans* and *Colpidium campylum*. All of these organisms were sterilized and established in pure culture in this laboratory and remain available to other investigators who may be interested in them for experimental purposes.

Material and Methods

Isolation and Sterilization

Tetrahymena geleii (strain W) was isolated from Mill Pond in Woods Hole, Massachusetts in July, 1939. It was sterilized in the migration-dilution apparatus described by Claff (1940) and established in pure culture.

Tetrahymena vorax is the strain previously described from this laboratory (Kidder, Lilly and Claff, 1940).

Glaucoma scintillans (strain A) was isolated from Mill Pond in July, 1939. It was sterilized in the migration-dilution apparatus of Claff and established in pure culture. Strain B was isolated from a freshwater stream near Providence, Rhode Island in May, 1940. It

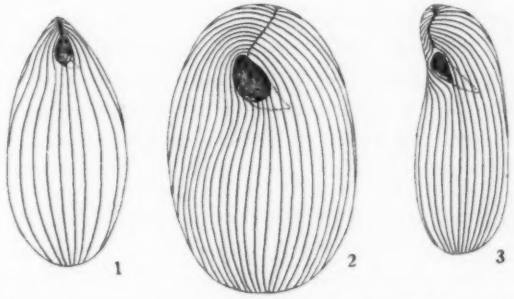

Fig. 1. *Tetrahymena geleii* (strain W). × 800. Total number of ciliary meridians = 17 — 19.

Fig. 2. *Glaucoma scintillans.* × 800. Total number of ciliary meridians = 35 — 40.

Fig. 3. *Colpidium campylum.* × 800. Total number of ciliary meridians = 27 — 30.

was sterilized and established in the same manner as strain A. Strain B will be discussed only in reference to adaptation to sterile conditions as it was not used in any of the other comparative studies.

Colpidium campylum was isolated from a freshwater stream near Providence, Rhode Island in September, 1939. It was sterilized by migration across a fluid-filled Petri dish (details of this method given elsewhere, Kidder, 1940) and established in pure culture.

Description of Species

Because of the confusion which has resulted from lack of adequate description of experimental material, three figures are presented which

show the diagnostic characteristics of those strains which were used and which have not been figured previously. These figures were prepared from opal blue treated material and indicate the distribution of the ciliary lines and the position of the mouth. Figure 1 is of *Tetrahymena geleii* (strain W) and corresponds almost exactly to the figures given by Furgason (1940) for this species. Figure 2 represents *Glaucoma scintillans* and Fig. 3 *Colpidium campylum*. No figure is given of *Tetrahymena vorax* as descriptions have been previously presented (Kidder, Lilly and Claff, 1940).

Studies of *Glaucoma scintillans* and *Colpidium campylum* have been made using the silver technique of Klein (1926) and the relief method of Bresslau (1922). Comparisons with slides prepared from different strains which were used in a previous study (Kidder and Diller, 1934) show that the present organisms are the same species as those reported at that time.

Conditions of Experiments

All qualitative studies were made from cultures grown in the specially designed Pyrex flasks described in detail elsewhere (Kidder, 1941). Organisms were counted by the direct method after appropriate dilutions. Qualitative observations were carried out on material grown in Pyrex test tubes.

Incubation of all experimental cultures was at 27° C. $\pm$ 0.2°.

Except in the experiments designed to test the effects of the age of the inoculum, all cultures were started from logarithmic phase ciliates. For uniformity the following ages of inocula were always used: *Tetrahymena geleii* (strain W)—18 hours; *T. vorax*—24 hours; *Glaucoma scintillans* and *Colpidium campylum*—48 hours.

Sterility tests on solid and in liquid media, incubated at room temperature and at 37° C., were carried out according to the methods outlined in previous studies of this series (Kidder and Stuart, 1939; Kidder, Lilly and Claff, 1940; Dewey and Kidder, 1940; Kidder, 1941) and, unless otherwise stated, all cultures were bacteria-free.

Method of Evaluation of Data

Attention should be called to an important point regarding the presentation and evaluation of data. The method often employed (Hall and Elliott, 1935; Hall, 1939; Hall and Schoenborn, 1939; etc.) of comparing the final concentration of cells (X) to the initial concentration (Xo) and expressing the result as the ratio X/Xo may lead to erroneous conclusions. The time selected for the final concentration count is arbitrary and may represent a point on the growth curve beyond

the cessation of logarithmic growth. No information is obtained regarding the activity of the cultures during the earlier phases of growth. The same criticisms apply to the method developed by Elliott (1939) where total protoplasmic volumes are compared, unless estimations are made in the early stages of the growth of the cultures. Therefore it seems not only desirable but necessary to follow the growth of cultures by taking numerous samples at regular intervals. The culture flasks used in these experiments were designed for such a procedure (Kidder, 1941).

Experimental Results

Physical Condition of Medium

Tetrahymena geleii (strain W) and *T. vorax* are both able to utilize dissolved proteins. This fact was immediately apparent upon the initial sterilization. They began rapid reproduction when placed in any of the standard peptone media or in Difco yeast extract. The addition of particles to such media did not increase the growth rate or the yield. These ciliates correspond to the other strains of *Tetrahymena,* therefore, in their ability to grow and reproduce in dissolved materials. Evidence is still lacking regarding Lwoff's (1932) contention that saprozoic ciliates are able to take in polypeptides through the pellicle. We still do not know whether extracellular enzymes are released which might hydrolyse proteins. If it can be shown that no such proteolytic enzymes are released into the medium, then it seems fairly certain that nutritive materials, even in the dissolved state, enter food vacuoles by way of the cytostome. This conclusion would be justified when it is noted that at least five strains of *Tetrahymena* (tested by V. C. Dewey in this laboratory) have been found to exhibit perfectly normal growth characteristics in dissolved casein. It seems highly improbable that whole protein molecules could be absorbed through the pellicle.

Both *Glaucoma scintillans* and *Colpidium campylum* are dependent upon particles of nutritive materials. This fact was noted by E. and M. Chatton (1923) for *G. scintillans* when they were able to obtain growth on dead *B. coli* but not on dissolved proteins. Hetherington (1933) reports the establishment of *G. scintillans* in yeast autolysate but the ciliates failed to reproduce beyond a few divisions and the cultures were presumably discarded.

When *Glaucoma scintillans* (strain A) was first sterilized single ciliates were placed in 2 per cent proteose peptone broth. After many days only a few divisions had occurred and it was apparent that the medium was inadequate. Difco yeast extract (1 per cent) and liquid yeast autolysate (10 per cent) were no better. Those ciliates placed in par-

ticulate Yeast-Harris (Kidder, Lilly and Claff, 1940; Kidder, 1940), however, reproduced quite rapidly while those placed in a mixture of Yeast-Harris and proteose peptone yielded thriving cultures (Fig. 4). Yeast-Harris or the mixture with proteose peptone which had been rendered particle-free by filtration gave no growth. It was later found that strain B and *Colpidium campylum* likewise require particles in the me-

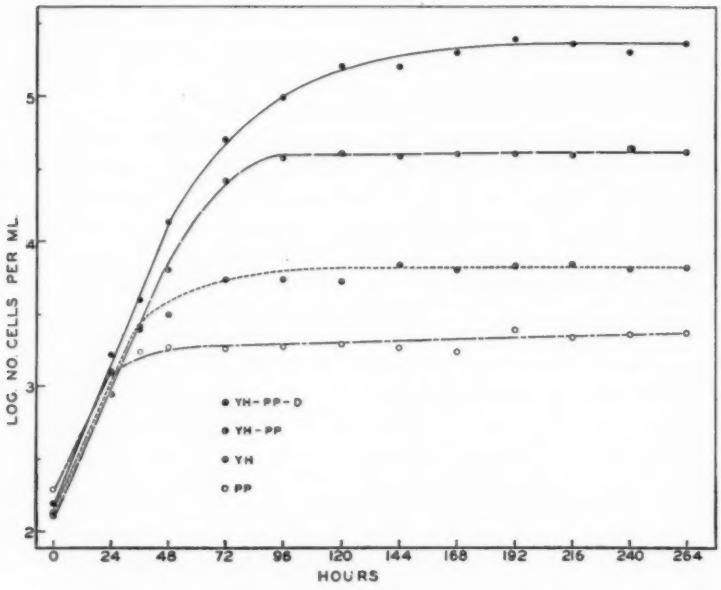

FIG. 4. *Glaucoma scintillans.* Growth curves constructed from the average data of 5 separate experiments. YH = 1 per cent Yeast-Harris; PP = 2 per cent proteose peptone; YH — PP = 1 per cent Yeast-Harris plus 2 per cent proteose peptone; YH — PP — D = 1 per cent Yeast-Harris plus 2 per cent proteose peptone plus 0.5 per cent dextrose.

dium and it was in this way that pure cultures of these strains were established.

Glaucoma and *Colpidium* appear to possess feeding mechanisms which are stimulated to ingestion only by solid particles. The slight amount of growth obtained in proteose peptone broth can be accounted for by the few particles which are invariably present after sterilization. When these particles are used up reproduction ceases.

After considerable experimentation the following basic medium was

adopted as the best for general use in dealing with *Glaucoma* and *Colpidium*—

Brewers Yeast-Harris	10 grams
Pyrex distilled water	1 liter

This is brought to a boil and filtered, first through cotton and then through Schleicher and Schüll No. 595 filter paper. This does not remove the finer particles of the broken yeast cells and the resulting solution is slightly turbid. To this liter of 1 per cent Yeast-Harris is added 20 grams of Difco proteose peptone and the whole sterilized in the autoclave at 15 pounds pressure for 20 minutes. This forms the base for the other experimental materials or may be used without additions. The concentrations given appear to be near optimum for these two species of ciliates as both higher and lower concentrations were inferior for growth.

Chemical Condition of Medium

No experiments on the inorganic requirements are to be described here as all of the media used contain sufficient inorganic constituents for all species (Lwoff, 1932).

In all media used the peptones, proteoses and proteins offered an adequate source of nitrogen. For the two species of *Tetrahymena* it was possible to test the relative effectiveness of various types of protein products on growth rate and maximum concentration. Various yeast products were tested as a substitute for and in combination with the standard proteose peptone. One per cent Difco yeast extract, one per cent filtered Yeast-Harris and 10 per cent liquid yeast autolysate were used. The rate of reproduction (as calculated by the formula

$$g = \frac{t \log 2}{\log b \log a},$$

where $g =$ the generation time and $t =$ the time in hours during which the population has been increasing, $a =$ the number of cells per unit volume at the beginning and $b =$ the number of cells at the end of time, t) in the case of *T. geleii*, was slightly lower in all three types of yeast media than in proteose peptone (Table I). The generation time for *T. vorax* was more than doubled (as compared with 2 per cent proteose peptone) in both yeast extract and yeast autolysate and was somewhat greater in Yeast-Harris (Table I). Some product of yeast autolysis (present in both the extract and the autolysate) seems to inhibit the reproduction of this species.

In the above experiments the various yeast factors were presented along with the yeast proteins. There remained the possibility that some

TABLE I

Tetrahymena. Comparison of growth in protein media. Average of four experiments.

Medium	Generation time in hours	
	T. geleii (strain W)	*T. vorax*
1 per cent yeast extract	3.51	7.07
10 per cent liquid yeast autolysate	3.34	7.90
1 per cent Yeast-Harris (particulate)	3.65	4.61
2 per cent proteose peptone	2.78	3.54

of these factors might stimulate growth if more adequate protein products were present. Consequently a "yeast vitamin concentrate—Harris" which is practically free of native protein was added to a basic medium of proteose peptone. Various concentrations were tested on *Tetrahymena geleii* (strain W) and the results are given in Table II.

TABLE II

Tetrahymena geleii (strain W). Comparison of growth after the addition of various concentrations of Yeast Vitamin Concentrate (Harris) to a basic medium of 2 per cent proteose peptone plus 0.5 per cent dextrose. Average of two experiments.

Percentage Yeast Vitamin Conc.	Generation time	Population per ml. at end of log. phase	Maximum yield
	hours		*cells/ml.*
0	3.37	48000	310000
0.025	3.14	52000	330000
0.05	2.95	67000	380000
0.1	2.70	70000	400000
0.2	2.74	54000	365000

The reproductive rate increased with the concentration up to 0.1 per cent but was slightly lowered at 0.2 per cent. The addition of yeast concentrate consistently lowered the reproductive rate of *T. vorax*.

The addition of yeast vitamin concentrate to the particulate medium used for *Glaucoma* and *Colpidium* had no significant effect up to a concentration of 0.2 per cent although higher concentrations caused inhibition of growth. These observations are of little significance, however, as the basic medium must contain rather high concentrations of the yeast factors.

The addition of a separate source of carbon to the basic proteose peptone medium for *Tetrahymena* and the particulate medium for *Glaucoma* and *Colpidium* increased the length of the logarithmic phase and the maximum yield of all species except *T. vorax*. As was mentioned in the initial report on *T. vorax* (Kidder, Lilly and Claff, 1940) dextrose

TABLE III

Tetrahymena vorax. Comparison of growth after the addition of various concentrations of dextrose to a basic medium of 2 per cent proteose peptone. Average of four experiments.

Percentage dextrose	Generation time	Population per ml. at end of log. phase	Maximum yield
	hours		*cells/ml.*
0	3.55	10000	70000
0.5	4.60	7000	42000
1.0	5.01	5500	32000
2.0	9.56	890	6000

decreases the division rate in direct proportion to its concentration (Table III) and the maximum yield is lowered in the same manner.

Experiments were conducted to test the ability of the four species of ciliates to ferment some of the more common carbohydrates. One polysaccharide (Difco soluble starch), four disaccharides (Difco saccharose, Difco maltose, Difco lactose and Difco cellobiose), three monosaccharides (Difco dextrose, Difco levulose and Difco galactose) and two pentose sugars (Special Chemicals arabinose and Difco xylose) were used. To the two types of basic media 0.5 per cent of the above carbohydrates and 0.02 per cent brom thymol blue were added. These media were dispensed in Pyrex tubes and sterilization was accomplished in the autoclave at 15 pounds pressure for 12 minutes. After cooling each type of media was inoculated with the four species of ciliates and the results were noted by the color change of the indicator after 96 hours in the case of *Tetrahymena* and 240 hours in the case of *Glaucoma* and *Colpidium.*

TABLE IV

Fermentation of carbohydrates. All carbohydrates added to basic protein media in 0.5 per cent concentrations. Medium contained 0.02 per cent brom thymol blue. Six experiments.

Carbohydrate	Organism			
	Tetrahymena geleii (strain W)	*Tetrahymena vorax*	*Glaucoma scintillans*	*Colpidium campylum*
starch	+	+	+	−
sucrose	−	−	−	+
maltose	+	+	+	+
lactose	−	−	−	−
cellobiose	+	+	+	−
dextrose	+	+	+	+
levulose	+	+	+	+
galactose	−	−	−	−
arabinose	−	−	−	−
xylose	−	−	−	−

The results of these fermentation experiments are given in Table IV. *Colpidium* alone failed to utilize starch and cellobiose. On the other hand, *Colpidium* was able to utilize sucrose while the other three species were not. None of the ciliates fermented galactose although Colas-Belcour and Lwoff (1925) report fermentation of this monosaccharide by their strain of *Tetrahymena* (*Glaucoma piriformis*). Galac-

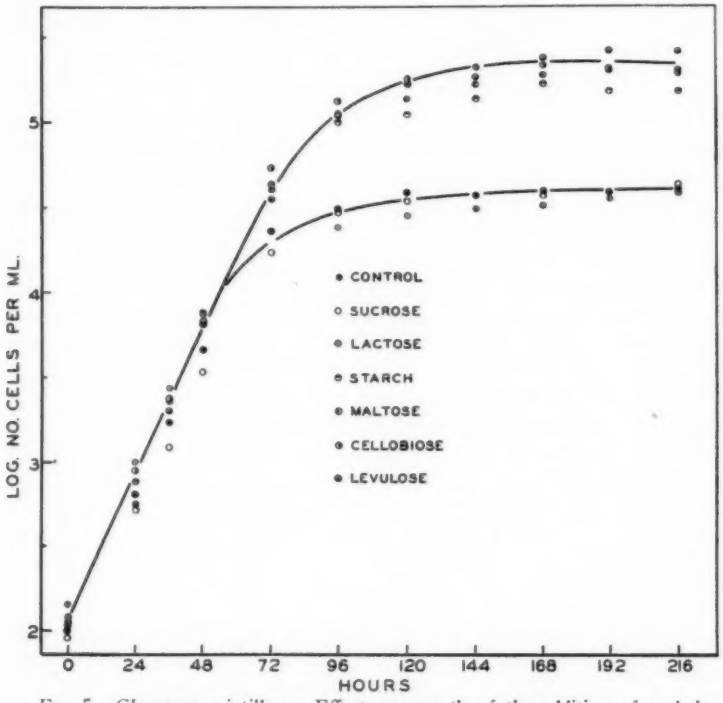

FIG. 5. *Glaucoma scintillans.* Effect on growth of the addition of carbohydrates to basic medium (1 per cent Yeast-Harris plus 2 per cent proteose peptone). All carbohydrates added in 0.5 per cent concentrations. Average of 3 experiments.

tose, arabinose and xylose so inhibited the growth of all the species that these carbohydrates were not used in the quantitative growth studies. All of the other carbohydrates were re-investigated in growth flasks and the cultures followed by frequent counts. The indicator was omitted but otherwise the media were as above.

The division rate, length of logarithmic phase and maximum yield were slightly increased by all of the carbohydrates fermented by *Tetra-*

hymena geleii (strain W). These increases were small but constant. No significant differences could be detected between any of the media containing fermentable carbohydrates. The acidity rose from an initial pH 6.8 to a final pH 4.8 in those flasks containing starch, maltose, cellobiose, dextrose or levulose while it fell in all others, including the control flasks, to pH 7.2.

Tetrahymena vorax, although it was able to ferment starch, maltose, cellobiose, dextrose and levulose (initial pH 6.8–final pH 5.4) was dis-

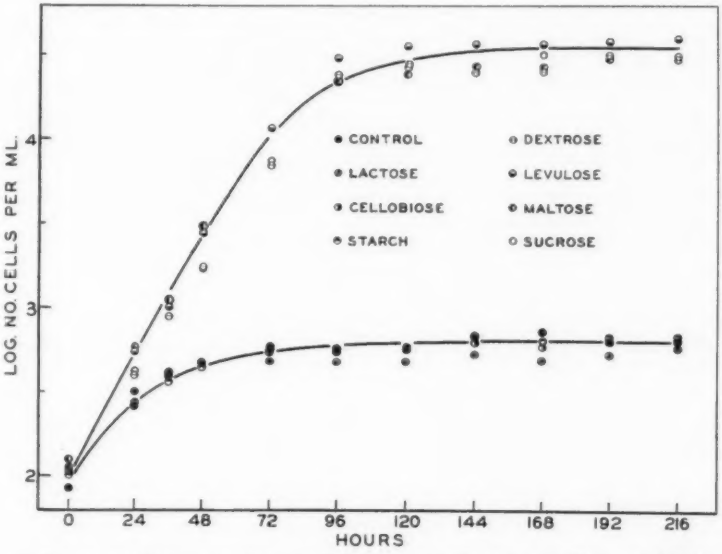

FIG. 6. *Colpidium campylum*. Effect on growth of the addition of carbohydrates to basic medium (1 per cent Yeast-Harris plus 2 per cent proteose peptone). All carbohydrates added in 0.5 per cent concentrations. Average of 5 experiments.

tinctly inhibited in its growth. Reproductive rate, length of logarithmic phase and maximum yield were decreased wherever fermentation occurred (see Table V for dextrose) and were unaffected (as compared with controls) in media containing carbohydrates which were not attacked (sucrose, lactose).

The most striking results of the addition of carbohydrates were found in the cases of *Glaucoma* and *Colpidium*. These results are given in Figs. 5 and 6.

The growth of *Colpidium* without carbohydrate was very slow and the maximum yield was low (not greater than 800 per ml.). The addi-

tion of 0.5 per cent dextrose, levulose, sucrose or maltose increased the rate of growth during the logarithmic phase and the maximum yield was increased to over 40,000 per ml. in some cases. For practical purposes, therefore, an additional source of carbon is a necessity for this ciliate.

The situation is somewhat different with *Glaucoma*. The division rate during the first 48 hours is not appreciably changed when a fermentable carbohydrate is added. Without a separate source of carbon, however, the end of the logarithmic phase is reached rather suddenly and

TABLE V

Summary of growth characteristics.

Organism	Medium	Optimum pH	Generation time	Population at end of log. phase	Maximum concentration per ml.
			hours		
Tetrahymena geleii (W)	2 per cent proteose peptone, 0.5 per cent dextrose, 0.1 per cent Yeast Vitamin Conc.	5.6–8.0	2.69	58000	395000
Tetrahymena vorax	2 per cent proteose peptone	6.2–7.6	3.52	12000	110000
Glaucoma scintillans	1 per cent Yeast-Harris, 2 per cent proteose peptone, 0.5 dextrose	5.6–6.8	7.37	40000	270000
Colpidium campylum	"	5.4	11.56	7200	41000

the curve flattens, the concentration (about 42,000 per ml.) remaining relatively constant thereafter for many days. The addition of sucrose or lactose has no significant effect upon the cultures, but the addition of dextrose, levulose, maltose, cellobiose or starch causes an increase in the length of the logarithmic phase, a long phase of negative growth acceleration and a final yield in excess of 200,000 per ml.

Optimum pH

A number of experiments were conducted to determine the optimum pH limits for the four species of ciliates. For these experiments three

types of media were used: 2 per cent proteose peptone for *Tetrahymena vorax;* 2 per cent proteose peptone plus 0.5 per cent dextrose for *T. geleii* (strain W); 1 per cent Yeast-Harris, 2 per cent proteose peptone, 0.5 per cent dextrose for *Glaucoma* and *Colpidium.* The pH was adjusted through a wide range of values (from pH 4.8 to pH 8.6) with HCl and NaOH.

The results of these experiments are contained in summary form in Table V.

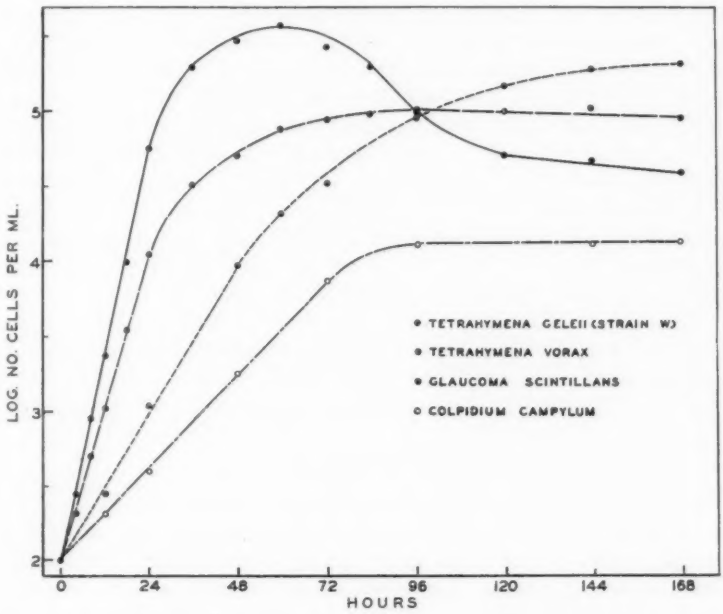

FIG. 7. Graphic comparison of the growth characteristics of four species of ciliates. Media as given in Table IX.

General Comparison of Growth Characteristics

Several interesting facts are brought out when the four species of ciliates are compared during the various phases of their growth. Figure 7 is a graph prepared from various experiments, each species growing under optimum conditions. A summary of data is given in Table V.

The growth rate of *Tetrahymena geleii* (strain W) is higher than any strain of ciliate in pure culture so far reported ($g = 2.69$ hrs.).

Unlike strain P (Phelps, 1935; 1936) and strain H (Kidder, 1941) of this species the negative acceleration period of strain W is quite long and the stationary phase is short. The concentration declines rapidly after the culture is approximately 60 hours old (initial inoculum of 100 cells per ml.) but the death rate decreases later (at about 120 hours) so that a concentration of 30,000–40,000 cells per ml. is maintained for many days.

Tetrahymena vorax grows at a regular rate ($g = 3.52$ hrs.) only during the first 24 hours. Thereafter a long negative growth acceleration phase ensues and the stationary phase is not reached until the culture is approximately 96 hours old. There is no decline in concentration, however, for many days so, in this respect, *T. vorax* resembles strains P and H of *T. geleii* (Phelps, 1935, 1936; Kidder, 1941).

In general the shape of the growth curve of *Glaucoma scintillans* is similar to that of *T. vorax*. The generation time during the logarithmic phase (first 48 hours of growth) is 7.37 hours. This rate gradually falls off and an extremely long negative growth acceleration phase takes place during which time the concentration increases from approximately 40,000 cells per ml. to over 200,000 per ml. The shape of this curve is reproducible under the conditions of these experiments.

The shape of the growth curve of *Colpidium campylum* is similar to that of strain H of *Tetrahymena geleii* (Kidder, 1941) although the growth rate is very low ($g = 11.56$ hrs.) as is the maximum yield (41,000 per ml.). This final concentration is maintained for many days.

Observations on Age and Size of Inoculum

As was stated earlier in this report, the ciliates used as inocula in the foregoing experiments were all taken from their logarithmic growth phases. Under these conditions no lag phase occurred. This corresponds to previous findings on controlled cultures (Phelps, 1935; Dewey and Kidder, 1940; Kidder, 1941). A lag phase invariably occurs if the ciliates which form the inoculum are taken from cultures which have passed the logarithmic growth phase. This statement holds for all four species used in the present study and has been reported for other strains and species (strains P and H of *Tetrahymena geleii*, Phelps, 1935; Kidder, 1941; *Perispira ovum*, Dewey and Kidder, 1940). The length of the lag phase increases, up to a certain point, in direct relation to the age of the parent culture.

The course of the growth is not dependent upon the size of the inoculum of *Tetrahymena geleii, T. vorax* or *Glaucoma scintillans*. Experiments on this point were conducted using large growth flasks (1

liter capacity) containing 500 ml. of media. Single ciliates were inoculated into these flasks. In order to insure the inoculation of active, single organisms they were first isolated into small containers made from the lower portion of shell vials. These containers had been previously placed in Petri dishes and sterilized. After the single ciliates had been isolated and checked under the dissecting binocular for number and activity, the whole container was lifted with sterile forceps and dropped into the culture flask. The initial inoculum is, in this case, 0.002 cells per ml. After growth has proceeded for sufficient time so that samples include enough cells for determination of numbers, the generation time is calculated and compared to the control flask which has received the usual inoculum of 100 cells per ml. No significant difference between the generation times in high and low inoculum cultures of the three species was obtained (Table VI). In no case did these single ciliates fail to establish perfectly normal cultures.

TABLE VI

Effect of size of inoculum. Figures represent generation time in hours. Volume of medium = 500 ml.

No. of cells per ml. inoculated	Organism			
	Tetrahymena geleii (strain W)	*Tetrahymena vorax*	*Glaucoma scintillans*	*Colpidium campylum*
100	2.69	3.52	7.37	11.66
0.002	2.71	3.48	7.41	18.25

Colpidium campylum did not give the same results (Table VI). In a number of the flasks inoculated with single ciliates no growth occurred. In those cultures which became established the generation time was significantly increased (18.25 hours as compared with 11.66 hours in the controls) and, what is more striking, the maximum yield was always very low (8,000 per ml. as compared with 40,000 per ml. in the controls). This species does not follow the same course as the other three and would seem to correspond to the reports of Robertson (1921–1927) on non-sterile organisms and of Mast and Pace (1938) on *Chilomonas*. Considering the general characteristics of *Colpidium*, however, I believe that there may be an explanation of the apparent "allelocatalytic" effect. Some substance or condition of the medium may be slightly detrimental to this ciliate. When large numbers of organisms are introduced no single cell receives a lethal amount of the toxic material. When a single ciliate is introduced into a large amount of medium it accumulates enough of the toxic substance to cause its death in some

cases or to injure it in others. When the injury is sub-lethal it nevertheless permanently affects the cells. The lowering of the maximum yield in single-cell-inoculated cultures which become established seems to favor this hypothesis.

Discussion

No complete analysis of the protein requirements of any ciliate is available at the present time due to a number of factors. Lwoff (1932) and Lwoff and Lwoff (1937) have obtained some data on their strain of *Tetrahymena geleii* (*Glaucoma piriformis*) but until all of the supplementary factors in relation to nutrition are more perfectly known this knowledge must remain incomplete. Neither *Glaucoma scintillans* nor *Colpidium campylum* appears to offer satisfactory experimental material for studies along this line. They both require particles. The particles obtained from powdered yeast cells are of nearly unknown chemical constitution. About all we can say concerning these particles is that they are very complex. To these particles appear to be adsorbed the molecules of proteoses and peptones necessary for the optimum growth. An attempt was made to substitute animal charcoal (Norit) for the powdered yeast. *Colpidium* failed to ingest these particles and while *Glaucoma* did ingest them at first (black food vacuoles), they later refused to do so and very little growth resulted. Various other inert materials which were tried proved no more successful. There may well be other types of particles (such as precipitated proteins, etc.) which could be substituted but no appreciable advantage would be gained. Casein, a well-known protein, was used successfully to supply the particles but the growth was never as good as in the Yeast-Harris medium, even though a filtrate of the Yeast-Harris was added.

It appears strange that *Tetrahymena vorax* is inhibited by some factor in yeast while the reproduction of *T. geleii* (strain W) is accelerated. This situation is also true of the fermentable carbohydrates. No answer to the question of these specific differences is available at present. It will be interesting to compare the supplementary requirements of these two species with the yeast factor question in mind.

Living organisms as food have been found to be a necessity for a number of species of ciliates (Phelps, 1934; Kidder and Stuart, 1939; etc.). This is not the case with *Glaucoma* and *Colpidium,* however. The most apparent difference between growth on a favorable bacterium and in pure culture is rate of reproduction. The living bacteria accelerate growth. This is not true in the case of *Tetrahymena,* where no species of food organism tested was as favorable for growth as the dissolved protein materials.

The observations on the carbohydrates are interesting in showing specific differences in enzyme production. While all four species of ciliates used in this investigation produce an amylase and a maltase, none of them produce lactase. *Colpidium campylum* stands alone in producing invertase and failing to produce cellobiase. All species ferment dextrose and levulose and fail to ferment galactose, arabinose and xylose.

With the exception of galactose and the pentose sugars, the carbohydrates which were not fermented did not influence the growth of any of the ciliates, although Elliottt (1935) reports some cases where acceleration of growth resulted without acid fermentation. These cases, however, must be questioned as he calculated acceleration by yield after a given time (usually 72 hours). The reason for questioning the validity of this method has been given in a previous section of this report.

Galactose, arabinose and xylose were found to be inhibitory to all four species of ciliates. Elliott (1935) reports inhibition of *Tetrahymena geleii* (strains H and E) by galactose, while Colas-Belcour and Lwoff (1925) record the fermentation of galactose by their strain of *T. geleii* but give no data regarding growth.

In the experiments designed to test the effect of the initial pH of the medium upon the growth of the ciliates investigated there was no indication that two optima exist as was reported by Elliott (1933) for his strain of *Tetrahymena geleii.* In fact, there were no significant differences in generation time, length of logarithmic phase or maximum yield over a wide pH range in the case of *T. geleii* (strain W), *T. vorax* or *Glaucoma scintillans. Colpidium campylum* reproduces faster, for a greater length of time and to greater final concentrations when the pH of the medium is low (pH 5.4).

No data are available from these experiments as to the factors which limit the period of maximum reproductive rate or cause the death of the organisms during the later stages of the cultures. It should be pointed out that the growth characteristics given are valid only under the conditions outlined and might well be changed somewhat by varying these conditions. The accumulation of volatile products of metabolism, such as CO_2, or the reduction of O_2 tension could be largely overcome by aeration. Phelps (1936) found that aeration increased the length of the logarithmic phase of *Tetrahymena geleii* (strain P) but did not alter the generation time in the early stages of growth.

A point of some interest which should be brought out is what Elliott (1933) and Johnson (1935) called "acclimatization." These authors report the necessity for gradually reducing the number of bacteria in the process of sterilizing their ciliates (*Tetrahymena*). These observations

were not confirmed on the ciliates used in this study. In every case establishment after complete sterilization followed immediately upon the presentation of an adequate medium. Another type of acclimatization was noted, however, in the case of *Glaucoma scintillans*. The growth rate (strain A) increased steadily during the first three months of sterile culture. The first calculations were based upon cursory data so this point was checked with strain B. One week after its initial isolation (May 27, 1940) growth flasks were inoculated and the generation time during the logarithmic phase was determined and found to be 12.21 hours. Cultures started June 10 grew more rapidly (generation time 10.64 hours) while those started on July 12 and September 20 were increasingly rapid (9.81 hours and 8.98 hours, respectively). Strain B, therefore, repeated what had been noted for strain A and although this strain does not reproduce as rapidly as strain A, even after four months, the same tendency of gradual adaptation to the sterile medium is shown.

Summary

1. The growth characteristics of four species of holotrichous ciliates (*Tetrahymena geleii, T. vorax, Glaucoma scintillans* and *Colpidium campylum*), grown in pure culture, are given.

2. The two species of *Tetrahymena* are able to utilize dissolved nutritive materials while *Glaucoma* and *Colpidium* are dependent upon particulate materials in the media.

3. The growth of *T. geleii* is slightly accelerated by some factor in yeast and by the presence of fermentable carbohydrates (dextrose, levulose, maltose, cellobiose and starch) while inhibition of the growth of *T. vorax* results when these materials are present.

4. The maximum yield of *Glaucoma* and *Colpidium* is greatly increased by fermentable carbohydrates.

5. *Colpidium* fails to ferment cellobiose but, unlike the other three species, does ferment sucrose.

6. Galactose, arabinose and xylose, while not fermented by any of the four species of ciliates, inhibit the growth of all.

7. The optimum range of pH values for *T. geleii* (strain W) is wide (pH 5.6 — pH 8.0); *T. vorax* is slightly more limited (pH 6.2 — pH 7.6); *Glaucoma* is limited to the acid range (pH 5.6 — pH 6.8), while *Colpidium* grows best at pH 5.4.

8. In the cases of *Tetrahymena geleii, T. vorax,* and *Glaucoma scintillans* when single ciliates from the logarithmic growth phase are inoculated into 500 ml. of media (initial inoculum = 0.002 cells per ml.) there is no lag phase and the generation time is not reduced (as compared with controls).

9. Single *Colpidium campylum* inoculated into 500 ml. of media often die. When a culture is established the generation time is longer and the maximum yield is smaller than when many cells are inoculated. It is suggested that these results are correlated with slight toxicity of the medium.

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THE EFFECT OF SAPONIN ON THE OSMOTIC HEMOLYSIS OF CHICKEN ERYTHROCYTES[1]

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I

Although the hemolysis of erythrocytes by lytic agents such as saponin has received much attention, few investigators have studied the effect of these agents prior to hemolysis. Ponder (1937) reported a decrease in the fragility of rabbit erythrocytes which had been exposed to sub-lytic concentrations of lysins. This effect, he believed, was similar to the action of narcotics (cf. Jacobs and Parpart, 1932). Davson and Danielli (1938) reported that saponin caused no loss of K^+ from erythrocytes in either sub-hemolytic or hemolytic concentrations.

The present experiments were performed to determine what effect saponin would have on the penetration of small molecules prior to the time that the membrane became permeable to the hemoglobin molecule.

II

Chicken blood was obtained by cardiac puncture and then defibrinated. One half a cc. of blood suspended in 5 cc. of Ringer-Locke required approximately 0.2 cc. of 1 per cent saponin in Ringer-Locke to cause slight hemolysis in two hours. A control suspension was similarly prepared, omitting the saponin. The slight difference in total volume (0.2 cc. in 5.5 cc.) did not introduce any error, since the method used to measure permeability is not influenced by variations in the number of cells within a wide range.

In this way stock suspensions of control and experimental cells were prepared. In most experiments 0.2 cc. of these suspensions were added to 10 cc. of an isosmotic solution of the substance whose rate of penetration was to be measured. Permeability, fragility, and swelling measurements were made at a temperature of $37° \pm 0.5°$ C., using the photronic cell technique usually employed in this laboratory (cf. Hunter, 1936; Hunter and Pahigian, 1940). In every experiment sufficient

[1] One of the authors (F. R. H.) is indebted to the American Association for the Advancement of Science for a grant-in-aid.

$NaHCO_3$ was added to insure complete clearing of the suspension (Stringer et al., 1940; Hunter et al., 1940). Permeability measurements were made until the experimental cells were found to hemolyze more rapidly than the controls when placed in the isosmotic solutions of penetrating substances. At this time the experimental solution was diluted with Ringer-Locke and centrifuged. In some of the experiments the supernatant fluid after centrifugalization was colorless and all of the packed cells were red, which indicated that the saponin had not caused any hemolysis. In others, the supernatant fluid was red and some of the cells were white. This indicated that the saponin had been allowed sufficient time to destroy the membranes of some of the cells and liberate the hemoglobin. Experiments in which this had happened served to indicate that the cells whose permeability was being studied had been exposed to hemolytic concentrations of the lysin, but the process had been stopped by removing the lytic agent before many of the cells had been hemolyzed. After the cells had been centrifuged, the supernatant fluid containing the saponin was discarded and the cells were resuspended in a volume of Ringer-Locke sufficient to give a suspension containing approximately the same number of cells per unit volume as the control. Additional washing with Ringer-Locke was unnecessary, as these cells would remain unhemolyzed for several days. These resuspended cells exhibited the same permeable properties that they had had immediately preceding the centrifugalization. The control cells were not centrifuged in every experiment, since this treatment had no marked effect on their permeability.

Some of the molecules penetrated the cells so rapidly at 37° C. that measurements could not be made using the photronic cell technique. In these cases the rate of hemolysis was measured by eye at room temperature (about 23° C.). The complete hemolysis curve could not be obtained in this way, but it was possible to make comparisons by measuring the times for a given percentage of the cells to hemolyze.

Permeability measurements were begun as soon as the control and experimental suspensions were prepared. A comparison of the effect of saponin on the permeability to glycerol and monoacetin was made using the photronic cell apparatus, while comparisons using the other method were made between various rapidly penetrating lipoid-soluble and insoluble molecules. Since it usually required at least one hour before the saponin produced any marked effect, there was sufficient time to make several measurements before the cells were centrifuged.

After the suspensions had been centrifuged and the cells resuspended in Ringer-Locke, hemolysis measurements were made using a number

of different substances. Centrifuged cells were used to obtain the swelling and fragility data. In some experiments, higher concentrations of saponin were used which produced slight hemolysis in 10–15 minutes. Cells obtained in this way gave the same results as those which had been exposed to lower concentrations of the lysin.

III

The data have been analyzed in several ways. In Table I are listed the average number of seconds required for 80 per cent hemolysis (one-half the total deflection) of the experimental and the control cells when placed in isosmotic solutions of the various substances studied. Since the change in hemolysis time depends on a number of factors such as the concentration of saponin, and the time allowed for its action, there

TABLE I

The effect of saponin on the osmotic hemolysis of chicken erythrocytes.

Substance	Average time in seconds for 80% hemolysis		Per cent change	Number of observations
	Control	Experimental		
*Water	18	7	−61.1	1
*Ethylene glycol	47	38	−19.2	10
*Diethylene glycol	75	39	−48.0	3
Triethylene glycol	130	87	−33.1	8
Urea	148	112	−24.3	6
Thiourea	193	148	−23.3	7
Glycerol	193	104	−46.1	38
Malonamide	868	273	−68.5	11
Erythritol	1125	292	−74.0	1
*Diacetin	34	23	−32.3	5
Monoacetin	144	63	−56.3	31
*Acetamide	38	22	−42.1	3
*Propionamide	38	22	−42.1	5

* Measurements made by eye.

is great variability observed in this value. To reduce this variability, only the figures obtained after the cells had been centrifuged are recorded. The differences in the percentage change which remain result from the fact that the saponin acted for a longer time in some experiments than in others. The table merely indicates that the permeability to a number of different substances has been greatly increased. The data for those substances which are starred were obtained from measurements made by eye. Figure 1 presents a representative control and experimental curve.

Having established the fact that treatment with saponin could increase the permeability of the cell membrane without causing hemolysis,

the data were analyzed in an attempt to demonstrate any differential effects. The possibility that an increase in permeability to lipoid soluble-molecules might appear first was considered. These data are presented in Table II. Once again there is considerable variability, but the evidence indicates that the experimental treatment increases the permeability

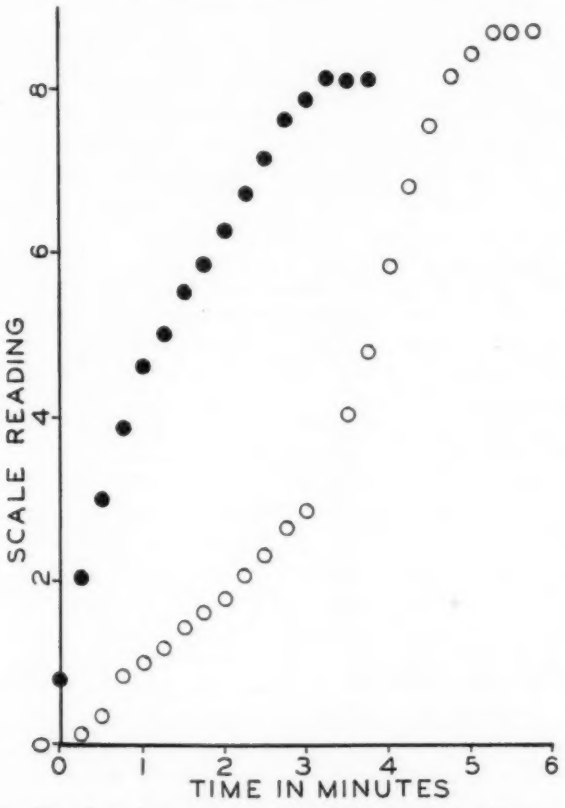

FIG. 1. The effect of saponin on the osmotic hemolysis of chicken erythrocytes in glycerol. ○—control; ●—experimental. (Scale reading represents degree of hemolysis.)

to lipoid-soluble and lipoid-insoluble molecules similarly. The time at which the increase is first noted and the degree of change are the same for the two types of substances.

Although the increase in permeability to lipoid-soluble and lipoid-insoluble molecules apparently occurs at the same time, it was thought

TABLE II

Data comparing the effect of saponin on the osmotic hemolysis of chicken erythrocytes in lipoid-soluble and lipoid-insoluble molecules after a short exposure to the saponin.

		Percentage change		
Date	Glycerol	Monoacetin	Ethylene glycol	Diacetin
3/6/40	−42.2	−62.2		
3/8/40	−13.8	−22.7		
3/27/40			− 7.2	−15.2
3/29/40			−18.3	−12.3
3/30/40	−11.1	− 4.5	+ 0.5	− 2.4
4/6/40			−16.2	−20.9
4/8/40	−18.0	− 9.5		

TABLE III

Data comparing the effect of saponin on the osmotic hemolysis of chicken erythrocytes in lipoid-soluble and lipoid-insoluble molecules after a long exposure to the saponin.

Glycerol			Monoacetin		
Time in seconds for 80% hemolysis		Percentage change	Time in seconds for 80% hemolysis		Percentage change
Control	Experimental		Control	Experimental	
200	50	−75.0	205	110	−46.3
215	142	−33.9	205	150	−26.8
175	24	−86.3	175	26	−85.2
195	115	−41.0	195	95	−51.3
195	95	−51.3	195	68	−65.1
131	18	−86.3	175	20	−88.6
125	50	−60.0	85	50	−41.2
106	80	−24.5	140	28	−80.0
165	100	−39.4	110	95	−13.6
154	152	− 1.3	130	112	−13.8
255	60	−76.5	155	38	−75.5
210	40	−81.0	130	90	−30.8
185	140	−24.2	130	40	−69.2
185	55	−70.3	130	110	−15.4
237	157	−33.8	175	25	−85.7
237	80	−66.2	205	135	−34.1
237	25	−89.5	205	40	−80.5
237	35	−85.2	205	23	−88.8
237	135	−43.0	146	82	−43.8
237	188	−20.7	146	130	−11.0
237	120	−49.4	146	70	−52.0
215	35	−83.7	127	13	−89.8
215	26	−87.9	127	13	−89.8
215	130	−39.5	127	37	−70.9
Average 192	86	−55.2	157	67	−57.3

possible that after the membrane had become considerably altered a difference in rate of penetration might appear. The data in Table III are presented as evidence on this point. These were obtained after the saponin had completed its action on the cell and had been removed by centrifugalization. The results indicate that under similar treatment the permeability to a lipoid-soluble molecule such as monoacetin is increased the same amount as the permeability to a lipoid-insoluble molecule such as glycerol.

TABLE IV

Data comparing the effect of saponin on the rate of penetration of a small and a large lipoid-insoluble molecule.

Glycerol			Malonamide		
Time in seconds for 80% hemolysis		Percentage change	Time in seconds for 80% hemolysis		Percentage change
Control	Experimental		Control	Experimental	
131	18	−86.3	670	204	−69.6
125	50	−60.0	578	126	−78.2
165	100	−39.4	805	644	−20.0
154	152	− 1.3	729	675	− 7.4
255	60	−76.5	1080	180	−83.3
237	157	−33.8	1080	75	−93.1
237	80	−66.2	920	540	−41.3
237	25	−89.4	920	210	−77.2
237	35	−85.2	920	83	−91.0
237	135	−43.0	920	110	−88.0
237	120	−49.4	920	150	−83.7
200	110	−45.0	730	390	−46.6
230	110	−52.2	810	330	−59.3
190	35	−81.6	570	60	−89.5
180	65	−63.9	480	65	−86.5
330	320	− 3.0	1190	1070	−10.1
340	315	− 7.4	1260	1080	−14.3
330	285	−13.6	630	270	−57.1
330	260	−21.2	690	210	−69.6
125	45	−64.0	400	120	−70.0
125	45	−64.0	640	150	−76.6
Average 221	120	−45.7	807	321	−60.2

Finally, a comparison was made of the effect of the experimental treatment on the penetration of a large molecule such as malonamide and a small molecule such as glycerol. In this case there appeared to be a consistent difference, but in order to be certain, an additional series of experiments was performed. All of the data are included in Table IV. The last ten sets of readings were from the second series of

experiments. These data indicate that the rate of entrance of malonamide is increased more by the experimental treatment than the rate of entrance of glycerol.

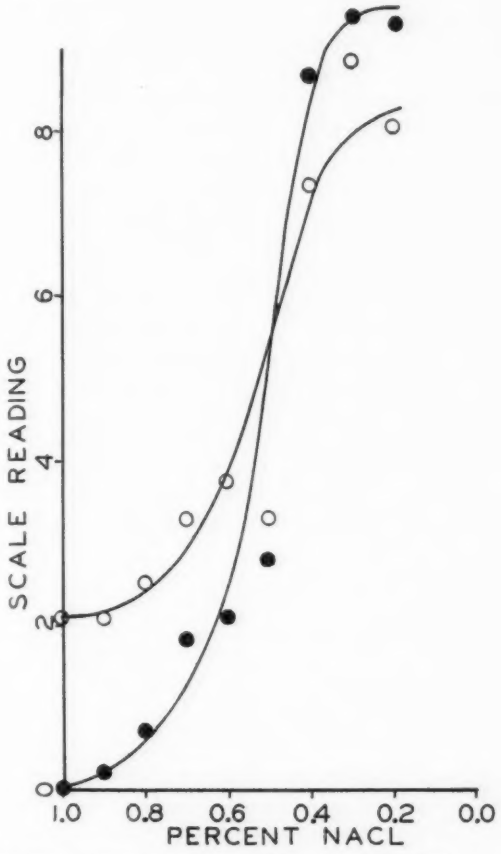

FIG. 2. The effect of saponin on the fragility of chicken erythrocytes. ○—control; ●—experimental (Scale reading represents degree of hemolysis.)

A decrease in the time for hemolysis does not necessarily indicate an increase in permeability (cf. Hunter and Pahigian, 1940). In order to test for a possible change in cell fragility, the following experiments were performed. Twenty cu.mm. of blood were added to 10 cc. of NaCl solutions of concentrations from 1.0 per cent to 0.2 per cent. The

pH of these solutions was adjusted by the addition of two drops of $NaHCO_3$ to each tube. A typical pair of curves is presented in Fig. 2. These data indicate that there is little, if any, change in the volume at which the experimental cells hemolyze.

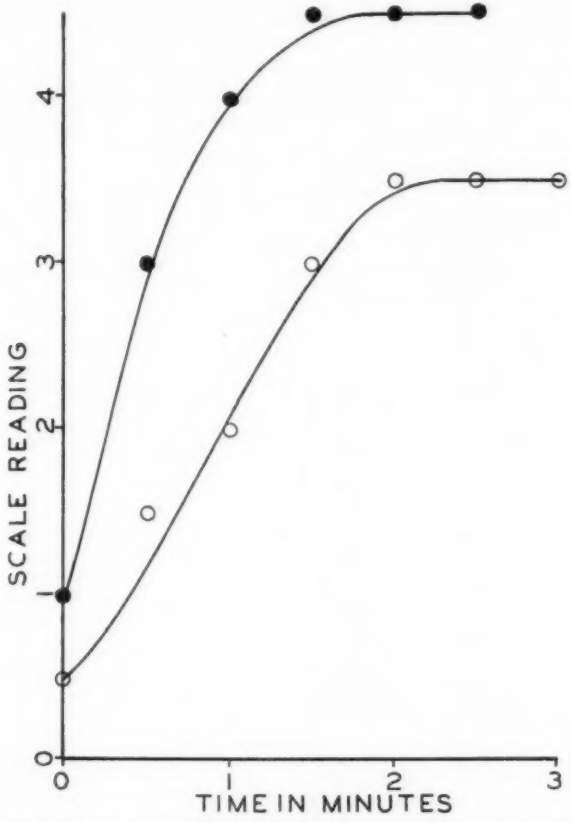

FIG. 3. The effect of saponin on the rate of swelling of chicken erythrocytes. ○—control; ●—experimental. (Scale reading represents amount of swelling.)

As a final test, swelling experiments were performed. Figure 3 presents a typical pair of curves obtained when control and experimental cells were added to an hyperosmotic solution of 0.3 M glycerol in Ringer-Locke. It can be seen that the experimental cells swell more rapidly than the controls. These data, then, definitely indicate an increase in permeability resulting from the experimental treatment.

IV

Schulman and Rideal (1937) presented evidence which indicated that the lytic action of saponin resulted from its reaction with cholesterol in the cell membrane. This would suggest that the experimental cells in the present investigation had altered membranes as a result of the action of saponin on some portion of the membrane in which lipoids were involved. The fact that the rate of penetration of lipoid-insoluble molecules, as well as that of lipoid-soluble molecules, was increased, would indicate that lipoid molecules in the membrane were in some way associated with the channels through which both types of molecules pass. A recent series of experiments by Ballentine and Parpart (1940), in which the effect of lipase on the cell surface was investigated, gave similar results. By making chemical analyses, these authors concluded that the lipase split fatty acids from the phospholipids in the cell surface. They suggested that these phospholipids were "an important structural unit in determining the rate of penetration in the aqueous channels."

As a result of the experiments in which lipase was used and those in which saponin was used, there is evidence to indicate that some of the lipoids in the cell membrane influence the passage not only of lipoid-soluble molecules but also of lipoid-insoluble molecules. It has been demonstrated that phospholipids are one type of molecule involved and the data contained in the present investigation suggest that cholesterol may be another.

Summary

1. Chicken erythrocytes exposed to low concentrations of saponin have their membranes altered.

2. By removing the saponin and resuspending the cells in Ringer-Locke, they will remain unhemolyzed for several days, even though the membranes have been altered.

3. These cells are more permeable to both lipoid-soluble and lipoid-insoluble molecules.

4. The penetration of both types of molecules appears to be affected equally.

5. The rate of penetration of a large molecule such as malonamide is increased more by this treatment than the rate of penetration of a smaller molecule such as glycerol.

6. The fragility of these cells is not increased by this treatment.

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THE ROLE OF TISSUES IN THE ANAEROBIC METABOLISM OF THE MUSSEL ANODONTA HALLENBECKII LEA

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Introduction

Lamellibranch mollusks possess the capacity for enduring anaerobiosis for a considerable time. Such a capacity is advantageous to tidal zone forms which are exposed to air at low tide, and likewise to freshwater mollusks which may have to endure low oxygen content of polluted water as well as exposure. Recognition of this peculiar ability has led to investigation of anaerobic metabolic processes of mussels. If the stream of water passing over the gills is cut off, the oxygen supply fails while carbon dioxide accumulates. The manner in which the mollusk deals with accumulating carbon dioxide has been the subject of several investigations.

Collip (1921) showed that the marine form *Mya arenaria* used calcium to buffer carbon dioxide. Dotterweich and Ellsner (1935) showed that in the freshwater mussel *Anodonta cygnea* most of the carbon dioxide formed during anaerobiosis entered into combination with calcium to form calcium bicarbonate. A small amount was buffered by calcium proteinate. They concluded that in general calcium in the shell of mollusks may be utilized as an alkali reserve.

Recent investigation by Dugal and Irving (1937) indicated that tissues as well as body fluids are involved in adjustment to oxygen lack. Mantle tissue of *Venus mercenaria* was found to accumulate carbon dioxide and calcium just as did mantle cavity fluid.

The work reported in the present paper was an investigation of the adjustment of a freshwater mollusk to a disturbance of the acid-base balance resulting from anaerobiosis. Particular reference was made to the rôle of mantle and gill tissues in this adjustment. Determinations of the carbon dioxide content gave results which indicated that mussel tissues were able to buffer carbon dioxide. The relation of calcium to the buffering process was studied. Observations were made on the oxygen consumption of tissues taken from asphyxiated animals. Evidence of an oxygen debt was found, showing that dissimilative processes were continuing through the period of anaerobiosis.

Materials and Methods

Animals used were freshwater mussels taken in the vicinity of Durham, N. C. They were identified by Dr. Henry van der Schalie of the University of Michigan Museum of Zoölogy as *Anodonta hallenbeckii* Lea.

Control animals were kept in tanks of running water. In this situation the valves remained open, allowing a constant stream of water to pass over the gills. Experimental animals were removed from such tanks and placed in a refrigerator with an air temperature of 6 to 8° C. At this temperature clams survived about a month. When removed from water *Anodonta* closed the shell valves. In this position exchange of gases between animal and environment was impossible. Any opening of the shell was accompanied by leakage of fluids from the mantle cavity. Leaking animals were not included in the experiments.

Tissues used were gill, mantle, and kidney tissue. Some observations were made on pallial muscle and foot muscle.

The rate of oxygen consumption of gill, mantle, and kidney tissue was measured in a standard Warburg apparatus. Tissue samples weighing about 0.1 gram were suspended in a salt solution containing 0.153 per cent NaCl. Absorption of carbon dioxide was accomplished with 20 per cent KOH. The temperature was held at 25° C. Measurements were made over a period of sixty minutes.

Carbon dioxide content of gill and mantle was determined by an adaptation of the Van Slyke manometric method for the determination of blood gases. The gas burette of the apparatus was modified from that described by Ferguson and Irving (1929). A ground joint at the lower end of the extraction chamber allowed the introduction of tissue. A weighed sample of tissue was placed in the extraction chamber, the burette put in place, and the joint made secure. Carbon dioxide was liberated by 0.1 N HCl introduced through the upper stopcock. Usually complete extraction required 45 minutes of shaking. Carbon dioxide was absorbed with air-free 1.5 N NaOH. The values P_1 and P_2 and the correction factor, c, were determined in the usual way.

Conversion of the observed pressure of carbon dioxide into cubic centimeters of gas was made according to the formulae modified for use with tissue samples by Ferguson and Irving (1929). Values for specific gravity were necessary for the conversion formulae. These values as determined were: for mantle, 1.04; for gill, 1.12.

Care was taken to maintain constancy in the method of obtaining and weighing tissue samples. It is felt that the values for carbon dioxide content are comparable, although they may not be absolute.

Calcium content of clam tissues was determined from samples digested in a mixture of concentrated nitric and perchloric acids. Calcium was precipitated from the digest as oxalate, redissolved and titrated with permanganate.

Results

Oxygen Consumption

Respiration of tissues from aerobic and anaerobic animals was compared. The results are given in Table I. The values for control animals are based on four determinations. They agreed closely. There were wider differences in the determinations on tissues from anaerobic animals. These values have been arranged by length of anaerobic period, and also averaged into one value for asphyxiated animals.

Table I

Oxygen uptake of gill, mantle, and kidney tissue. Values are averages of two to four determinations and represent cubic millimeters of oxygen consumed per hour per milligram dry weight of tissue at 25° C.

Days out of water	Kidney	Mantle	Gill
14	4.70	1.32	.486
10	3.66	1.34	.766
8	2.69	1.24	.652
6	2.82	1.45	.758
4	3.24	1.02	.660
2	2.82		.573
Average	3.32	1.27	.649
Average of controls	2.11	1.02	.421

Tissues removed from asphyxiated animals consumed more oxygen per hour per unit weight than did tissues from control animals. This was true for the first hour after removal. Determinations were not carried beyond this point. It is therefore impossible to make any calculation of the total extra oxygen required. However, the increase noted suggests the paying off of an oxygen debt incurred during anaerobiosis.

The respiration rates are referred to dry weight of tissue. It was found that mussel tissues varied in water content from one individual to another. There was no evidence of a correlation between dry weight and anaerobic period. The observed percentages dry weight as averaged from a large number of samples studied are given below:

mantle	3.9
kidney	8.6
gill	24.0

It is interesting that the rate of oxygen consumption of kidney tissue was much higher than that of other tissues studied. According to Holmes (1937), the high rate of respiration of mammalian kidney tissue is due to osmotic work done by excretory cells. Probably a similar explanation fits the case of mussel kidney.

Carbon Dioxide Content

Results of the determination of the carbon dioxide content of gill and mantle are given in Table II. The following points are to be noted:

TABLE II

Carbon dioxide content of mantle and gill. Values are expressed as cubic centimeters of gas at standard temperature and pressure and equivalents of carbon dioxide in one hundred grams fresh tissue. Averages of several determinations are represented.

Days out of water	Mantle	Gill	Mantle	Gill
	cc./100 gr.	*cc./100 gr.*	*equiv./100 gr.*	*equiv./100 gr.*
0	25.0	322	0.0022	0.0287
1		399		0.0356
2	32.2	372	0.0028	0.0332
3	35.0	369	0.0030	0.0328
4	32.0	405	0.0028	0.0376
6	34.4	430	0.0030	0.0392
8	43.6	455	0.0038	0.0406
10	44.2	487	0.0038	0.0432
12		499		0.0444
14	47.7	512	0.0042	0.0456

1. Gills contained approximately ten times as much carbon dioxide as did mantles.

2. There was a steady increase in the amount of carbon dioxide accumulating in gill tissue during anaerobiosis.

3. Carbon dioxide accumulated in mantle tissue in proportion to the increase in gill tissue. The equivalents of carbon dioxide in mantle doubled during asphyxiation.

For purposes of comparison with the amount of calcium present, the values for carbon dioxide were converted into equivalents and are also given in Table II.

Calcium Content

It was found that the calcium content of the tissues studied did not vary significantly with the period of anaerobiosis. Averages from a large number of determinations are given below, expressed as milligrams of calcium per gram dry weight of tissue.

foot muscle	8	mg./gram tissue
pallial muscle	31	"
kidney	46	"
mantle	62	"
gill	175	"

Gill tissue contained a large amount of calcium as compared with other tissues. This may be correlated with the relatively high dry weight of gill tissue. The small amount of calcium found in foot muscle is surprising when considered with the other values.

By using the percentage dry weight of mantle and gill tissue it was possible to calculate equivalents of calcium per one hundred grams fresh tissue. These were found to be: for mantle, 0.0045; for gill, 0.21.

Discussion

Study of the functioning of animal tissue in buffering processes has not been investigated in many species. Dotterweich (1933) showed that the calciferous glands of earthworms were capable of giving up calcium to buffer carbon dioxide accumulating in body fluids. Banus and Katz (1927) found weak buffering by hind leg muscles of a cat. A similar effect was noted by Irving and Chute (1932) in muscle.

A buffer system in the tissues of *Anodonta* is indicated by a study of the carbon dioxide and calcium content of certain tissues. Gill tissue seems to be most active in this respect.

From the data given above, it is seen that one hundred grams fresh gill tissue contain 0.21 equivalents of calcium, and 0.0287 equivalents of carbon dioxide (see Table II). This proportion indicates that most, possibly all, the calcium is present in some form other than carbonate.

During anaerobiosis the carbon dioxide level rises, increasing to 0.0456 equivalents at 14 days. This increase is not accompanied by an increase in the hydrogen ion concentration. The hydrogen ion concentration of the tissue was measured colorimetrically, and was found to vary less than 0.05 from pH 6.8 for gill, 6.9 for mantle. Apparently the accumulating carbon dioxide is bound in some way so that an increase in hydrogen ions does not occur.

It was suggested by Dotterweich and Ellsner (1935) that a calcium-proteinate might act as an additional buffer in the fluid of *Anodonta cygnea*. In that system calcium carbonate was the principal alkali reserve. In the tissues of *Anodonta hallenbeckii* it would seem that calcium-proteinate, or some other combination of a weak acid with calcium, is the chief buffer, with the carbonate playing at the most a minor rôle.

In the case of mantle tissue 0.0045 equivalents of calcium are present in the normal mantle. Carbon dioxide increases from 0.0022 equivalents in the normal tissue to 0.0042 equivalents in the asphyxiated tissue. The calcium and carbon dioxide are then in a one-to-one ratio. This would indicate a more limited calcium reserve in mantle than in gill.

Dugal (1939) has shown that in *Venus* the calcium reserve may be augmented by calcium from the shell. Tissues of *Anodonta* maintain a steady calcium level.

Calcium is not only the chief component of the hard parts of mollusks but also forms a considerable portion of the alkali reserve. The same factors which govern the precipitation of solid calcium in the shell are responsible for the deposition of calcium in tissues. It is a point of interest that freshwater clams possess large deposits of calcium in their gills, and marine clams possess the larger deposits in mantle tissues (McCance and Shipp, 1933). There may be some correlation here with the fact that glochidia develop in the gill pouches of freshwater mussels and may derive calcium for their shells from the abundant supply available.

Jatzenko (1928) showed that certain freshwater mussels build up an oxygen debt during anaerobiosis. It is to be expected that individual tissues would also show such a debt. All activity does not cease when the clam is temporarily asphyxiated. Some of it continues. Ciliary action such as accounts for a great deal of the oxygen consumption of gill and mantle probably does decrease to some extent. Osmotic work which is characteristic of kidney tissue continues and may even increase during anaerobiosis. Data for individual tissues as presented in Table I show that oxygen consumption of mussel tissues is higher immediately after a period of asphyxiation than under normal conditions.

The source of energy for activities carried on during anaerobic periods is generally laid to a glycolytic process. However, there has as yet been no isolation of the tissue or tissues mainly responsible for the glycogen reserve. The problem of the energy source and its localization is a pertinent one to a complete explanation of the anaerobic metabolism of mussels.

Summary

Tissues of *Anodonta hallenbeckii* are capable of buffering carbon dioxide accumulating during anaerobiosis. Calcium compounds present in gill and mantle serve as an alkali reserve. During anaerobiosis carbon dioxide increased in the tissues studied while the hydrogen ion concentration remained constant. It is concluded that accumulated carbon dioxide was buffered by calcium present.

Gills contain large amounts of calcium which is present in some form other than carbonate.

Kidney tissue showed a very high rate of respiration. Mantle and gill showed low rates. After anaerobic periods the rate of respiration showed a tendency to increase. This may be taken as evidence that these tissues continued to do work during anaerobiosis.

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THE EFFECT OF THE CIRCULATION OF WATER ON THE DISTRIBUTION OF THE CALANOID COMMUNITY IN THE GULF OF MAINE[1]

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Damas (1905) has pointed out that the flow of water tends to dissipate local populations of pelagic organisms, and that the permanence of breeding stocks may be maintained by the existence of eddies. His predictions have been strikingly confirmed by hydrographic observations in the Norwegian Sea (Sømme, 1933). Along the margins of the Gulf of Maine the permanence of the stock of *Sagitta elegans* is correlated with the stability of the hydrographic conditions which exist in different regions (Redfield and Beale, 1940). Walford (1938) has indicated the importance of fluctuations in the circulation on Georges Bank to the fate of haddock eggs spawned in that region. These studies and that of the author (1939) on the population of *Limacina retroversa* emphasize the rapidity with which currents move pelagic organisms about within the Gulf. It becomes a problem whether the community of the basin of the Gulf is truly endemic, and by what mechanism a breeding stock is maintained within the Gulf. Sømme (1934) has discussed this question in regard to the copepod population of the Lofoten area.

Bigelow (1926), who has described the zoöplankton of the Gulf in great detail, considers that the species which form the bulk of the pelagic population are endemic in origin, breeding with sufficient regularity and abundance to maintain the local stock by local reproduction. From its dominating member, *Calanus finmarchicus,* he has referred to the population as the calanoid community.

We have measured the catches taken in the Gulf during a year-round survey and will attempt to explain the distribution of numerical abundance in terms of the pattern of currents obtained during the period of observation.

Data

The data employed in the present study were collected in the course of cruises made by the research vessel "Atlantis" during the years of 1933 and 1934. The dates of these cruises and the numbers of the

[1] Contribution No. 281 from the Woods Hole Oceanographic Institution.

stations occupied are given in Table I. Thirteen cruises were made in the course of fifteen months, with the result that 684 hydrographic stations in the Gulf of Maine and its adjacent waters were occupied. At no time did a period longer than two months elapse without observation. The routine hydrographic and chemical data are published in the Bulletin Hydrographique (1933, 1934).

A supplementary cruise was made in May, 1936 in order to confirm certain observations made during the primary survey.

In the course of the cruises standard vertical hauls were made with a 1.5 meter Heligoland net of No. 0 silk having 38 meshes to the inch (Künne, 1933). The net was hauled from a point near the bottom to the surface at all stations occupied, weather permitting. This type of haul was selected in preference to the oblique haul in the belief that the procedure could be carried out uniformly as a part of the routine duties

TABLE I

Cruise No.	Dates	Stations	Number
16 and 17	June 19–July 10, 1933	1643–1721	79
21	Sept. 2–Sept. 14, 1933	1741–1802	62
22	Oct. 17–Oct. 29, 1933	1803–1860	58
23	Dec. 2–Dec. 11, 1933	1861–1906	46
24	Jan. 8–Jan. 13, 1934	1907–1934	28
26	Mar. 21–Mar. 29, 1934	2019–2070	52
27 and 28	Apr. 17–May 13, 1934	2071–2164	94
29	May 21–June 3, 1934	2165–2215	51
31	June 25–July 1, 1934	2217–2236	20
34	Aug. 10–Aug. 11, 1934	2252–2259	8
37	Sept. 17–Sept. 27, 1934	2268–2303	36
55	May 14–May 19, 1936	2555–2583	29

of the ship's company. Unfortunately, it proved impossible to use the net in rough weather, so that data are lacking from many stations, particularly those made in the winter months. The yields of the successful hauls have been measured by collecting the plankton on filter paper in a Buchner funnel. Suction was continued until the preserving fluid ceased to flow, whereupon the "dry" plankton was introduced into a measured volume of fluid and the resulting increase in volume noted. The data so obtained were reduced to figures expressing the number of cubic centimeters of "dry" plankton under each square meter of the sea surface. Before filtering and measuring collections, any large gelatinous organisms were removed (*Salpa*, ctenophores, medusae) with the result that the measurements reflect primarily the abundance of the crustacean community.

Since most of the hauls with which we are concerned were made in

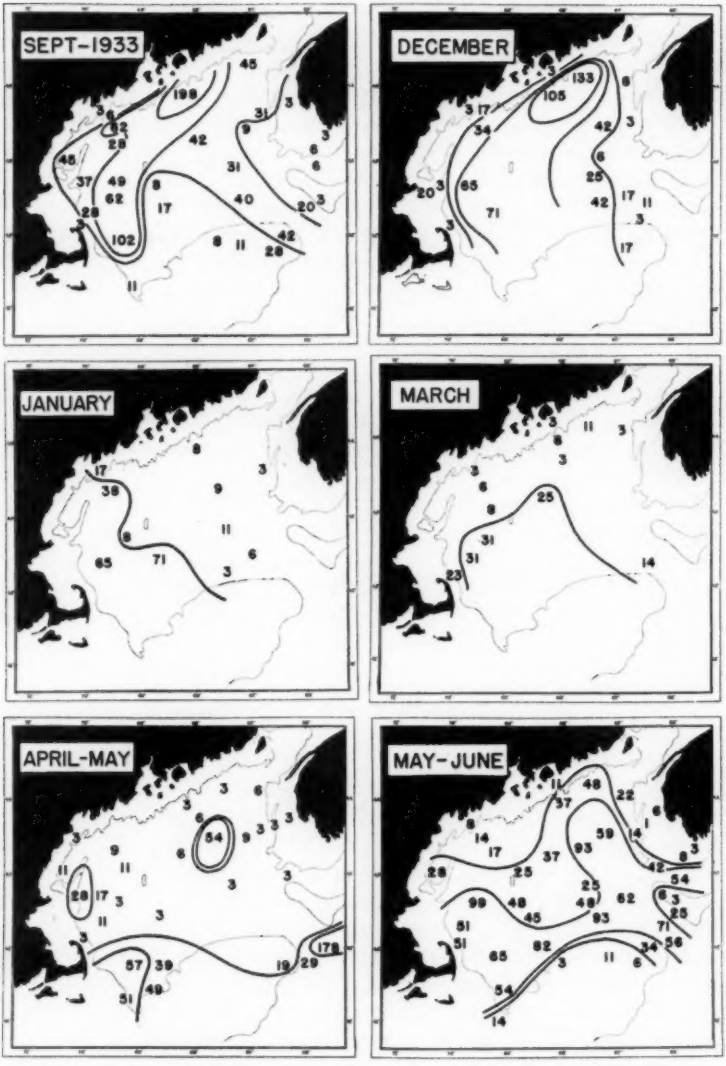

FIG. 1. Volumes of zoöplankton taken in vertical hauls between September, 1933 and June, 1934. Numbers represent the cubic centimeters taken per square meter of sea surface. Contour interval 25, 50, 100, and 200 cc. per square meter.

depths greater than 100 meters, above which level most of the population may be expected to occur, these figures are thought to express the density of population more precisely than numbers reduced to unit volume of water strained. The general character of the results is not altered by expressing the catch in terms of the yield per cubic meter.

The determination of the " dry " volume of the catch by the method of filtration and displacement yields smaller values than are obtained by the " wet " method of allowing the animals to settle in a calibrated container. In order that our results may be compared with those of Bigelow and others who employed the settling method, a number of samples have been measured by both methods. The wet method gave values on the average 4.9 times higher than the dry method, the ratios

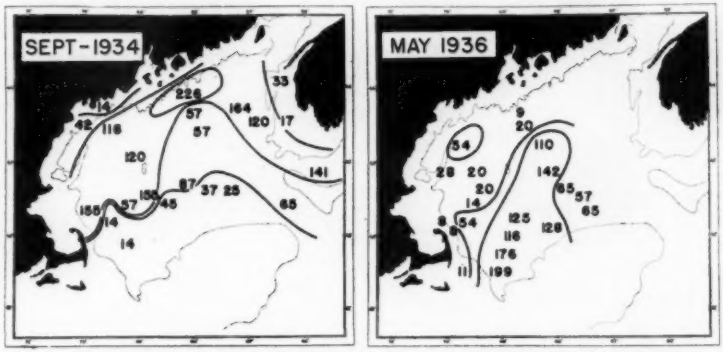

FIG. 2. Volumes of zoöplankton taken in vertical hauls in September, 1934 and May, 1936. Numbers represent the cubic centimeters taken per square meter of sea surface. Contour interval 50, 100, 200 cc. per square meter.

varying between 3.3 and 7. The ratio was smaller in the case of the larger samples, due perhaps to the tighter packing of large samples in the wet method and to the greater retention of water when large quantities of organisms are filtered off in the " dry " method.

THE SEASONAL DISTRIBUTION OF THE ZOÖPLANKTON POPULATION

The quantities of the catches obtained by vertical hauls, and their positions during the most complete periods of survey, are entered on the charts shown in Figs. 1 and 2. These charts show that the area of maximum abundance shifts its position with the season. From late summer until December the richest population is found in the northern portion of the Gulf, centering off Mount Desert. During the winter the center shifts to the west, coming to lie off the Massachusetts coast.

In late spring and early summer the richest catches were obtained along the southern margin of the Gulf, north of Georges Bank, extending from the offing of Cape Cod, eastward and northward toward the Bay of Fundy.

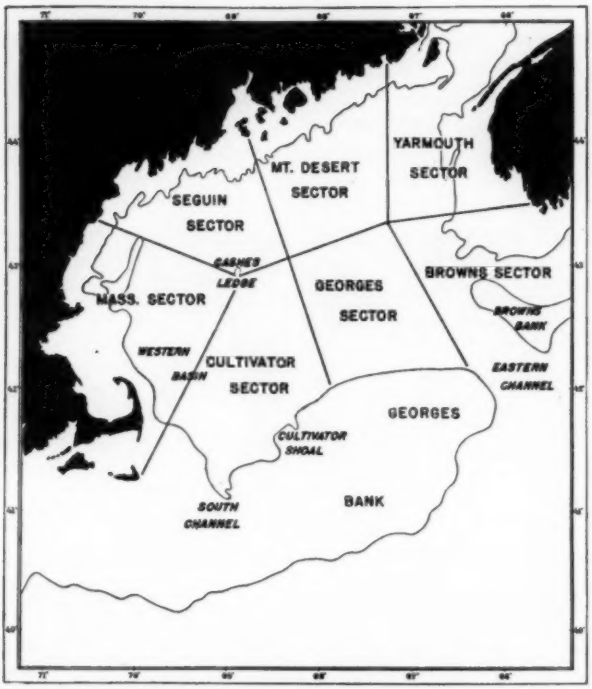

FIG. 3. Chart of the Gulf of Maine showing principal place names and the sectors into which the area is divided for analysis of population distribution. Contour encloses depths less than 100 meters.

In order to deal with the data statistically, the area of the Gulf has been divided into seven sectors as shown in Fig. 3. Each sector includes one of the principal lines of stations at which collections were regularly made. The quantities of plankton taken at each cruise in each sector have been averaged and the resulting number taken to represent the density of population in that sector at the time. While the data are frequently numerically inadequate, certain interesting regularities appear from its analysis.

Figure 4 shows the density of population in the various sectors at each principal period of survey. It presents graphically the shift in the center of population westward from the Mount Desert to the Massa-

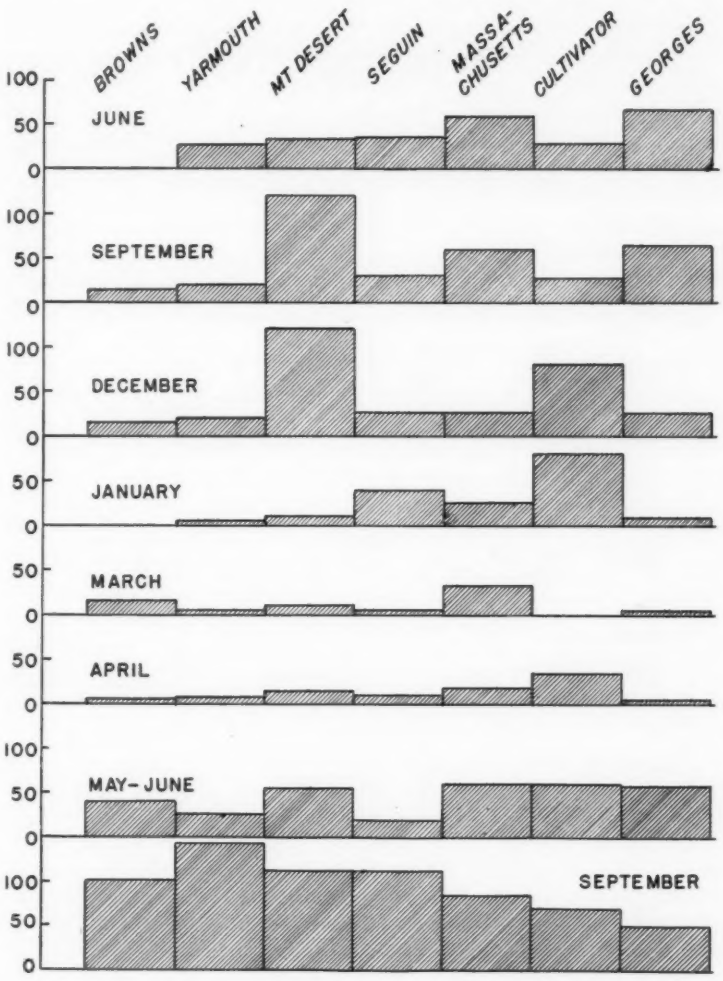

FIG. 4. The average catch in each sector of the Gulf of Maine during the period September, 1933 to September, 1934. Ordinates: cubic centimeters of zoöplankton per square meter of sea surface.

chusetts sector in the course of the winter and its extension along the southern sectors in May and June, followed by the reëstablishment of a maximal population in the northeastern sectors in September.

Figure 5 presents the same data in a form which brings out the sea-

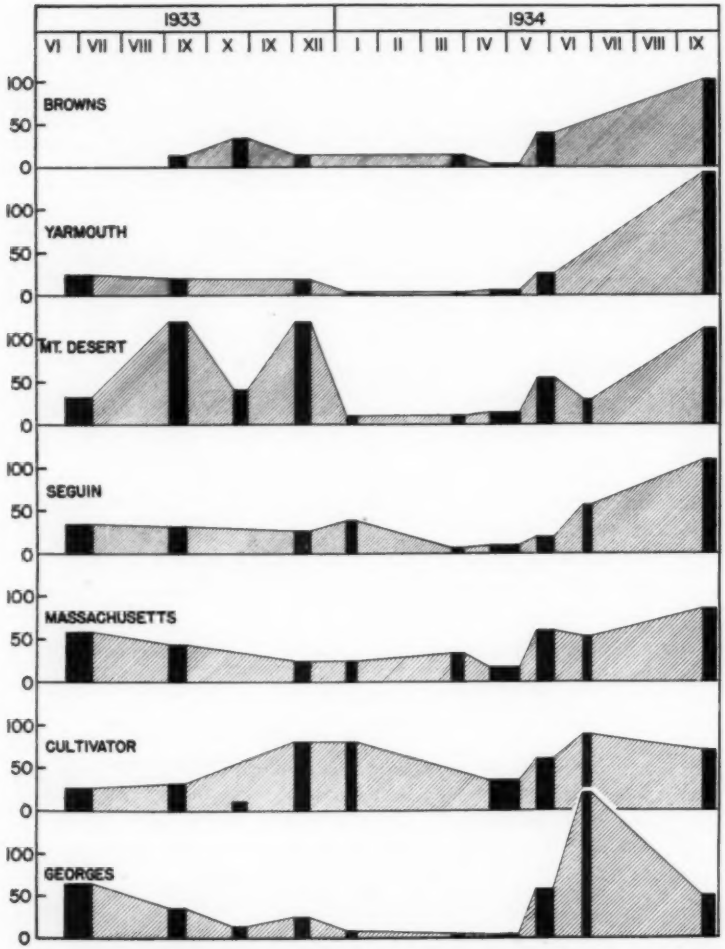

FIG. 5. Seasonal fluctuation of catch in each sector of the Gulf of Maine during the period June, 1933 to September, 1934. Ordinates: cubic centimeters of zoöplankton per square meter of sea surface. The black bars indicate the actual period occupied by each cruise.

sonal fluctuation in each sector. The sectors along the east and north sides of the Gulf are marked by a pronounced seasonal fluctuation—most extreme in the Mount Desert region. In contrast, the population is much more uniformly distributed from month to month in the Massachusetts and Cultivator sectors, which include the greater part of the western basin.

The general features of the distribution appear to recur from year to year, for our observations for September 1933 and 1934 show essentially similar patterns, as do also those for May–June, 1934 when compared with May, 1936. The seasonal distribution observed by Bigelow over a number of years is also in agreement. He found the quantitative fluctuations to be comparatively narrow from season to season in the waters of the western basin and considered the plankton in that part of the Gulf to be " rich " the year round. He reports the northern corner of the eastern basin, as well as the shallows off Cape Sable, to be the site of a wide seasonal fluctuation (Bigelow, 1926, p. 89).

The Circulation of the Gulf

The shift in the center of abundance of the zoöplankton population suggests that it is being borne about a great cyclonic eddy. We may consequently examine the nature of the circulation of the Gulf to see if it can account for the fluctuations in numbers in different places and to learn to what extent the calanoid community may be carried into and out of the Gulf by water movements.

The evidence marshalled by Bigelow (1927)—measurements with current meters, drift-bottles, temperatures, salinities, distribution of plankton, and dynamic calculations—can be harmonized with one type of dominant circulation only, a general anti-clockwise eddy around the basin of the Gulf. The demonstration of this, named by Huntsman (1924) and by Bigelow the " Maine " or " Gulf of Maine " eddy, with all it implies in its biological bearing, is perhaps the most interesting result of their joint explorations of the Gulf. Observations made during a series of years demonstrated that the center of the eddy shifted its precise location from summer to summer, and that marked seasonal variations in the circulatory scheme occurred. Observations of the velocity of the non-tidal drift of the surface made in shoal water about the margin of the Gulf indicated an average movement of seven miles per day, at which rate some three months would be required to complete the circuit of the eddy. No estimations were made of the velocity of the deeper layers.

The hydrographic data collected in the course of the cruises in 1933–34 have been analyzed by Dr. E. E. Watson, who has kindly per-

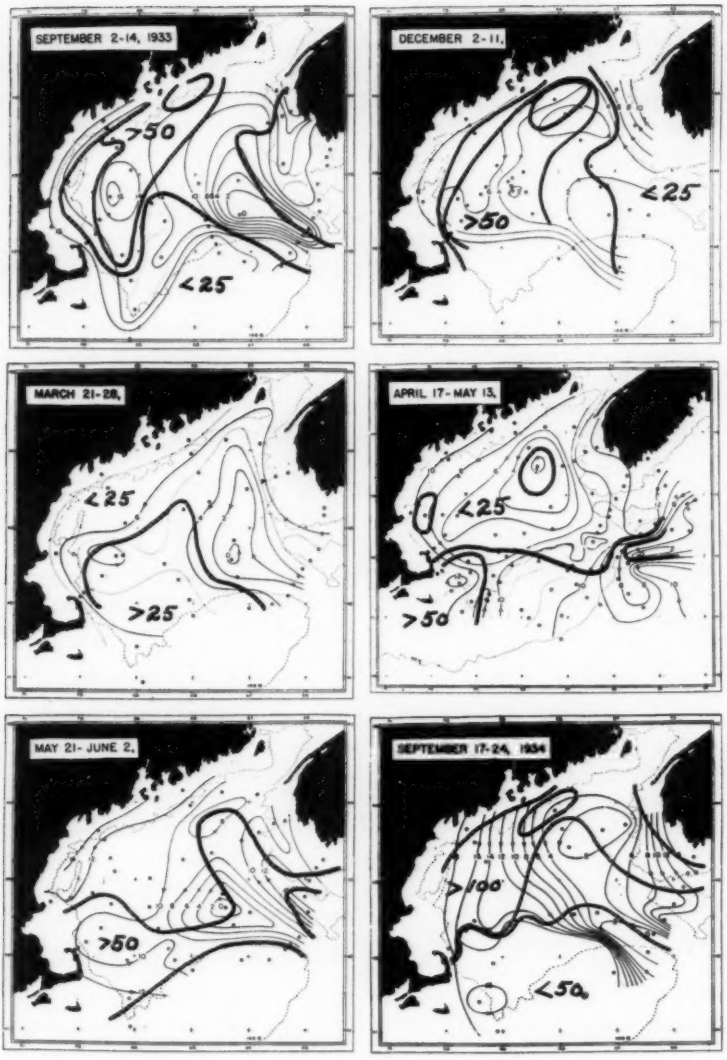

FIG. 6. Dynamic contour charts showing the theoretical circulation of the Gulf of Maine at the surface between September, 1933 and September, 1934. The heavy contours, taken from Figs. 1 and 2, indicate the relative density of population at the time of each cruise.

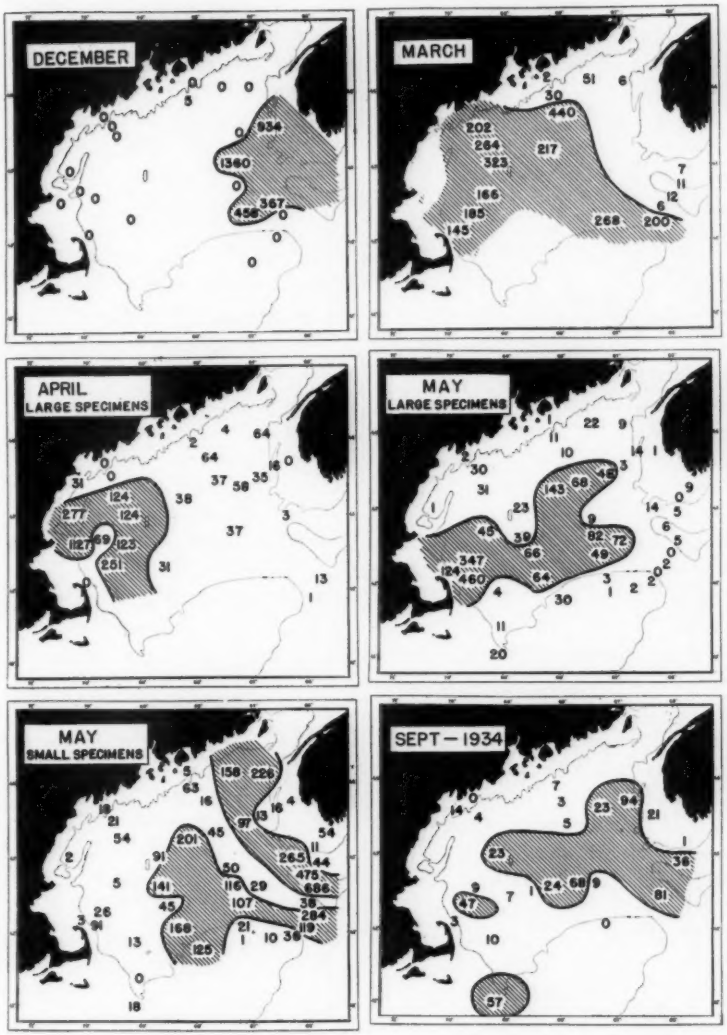

FIG. 7. The distribution of *Limacina retroversa* in the Gulf of Maine between December, 1933 and September, 1934. The numerals indicate the position and the numbers caught per haul. Compare Fig. 6 for corresponding current diagrams and the coincident distribution of the calanoid community.

mitted me to use some of his current diagrams in advance of the publication of his full report. This study has not only confirmed the more general conclusions of Bigelow, but gives the best available evidence of the actual character of the circulation at the time of our collections.

The zoöplankton population is not distributed uniformly in waters of various depth but tends on the whole to congregate in the upper 100 meters (Bigelow, 1926, p. 93). In many species, particularly of the numerically important copepods, there is a pronounced diurnal vertical migration which has been studied in the Gulf of Maine by Clarke (1933, 1934). At a station in the deep part of the Gulf, he found that *Calanus* and *Metridia* migrated to a depth of 120 meters or more during the daytime and moved upward to levels of from 6 to 42 meters at night. On Georges Bank *Calanus* was confined to the surface strata, undergoing very limited migration, but *Metridia* carried out an extensive vertical migration. Consequently the population cannot be identified exclusively with any particular layer and any attempt to correlate its distribution with the drift of the water is complicated by the undoubted migration of the animals to and from layers of different depth moving with different velocities and in some places without doubt in different directions. As a first approximation, however, it is reasonable to assume that considerable volumes of the more superficial water, unconfined in its movements by shoals, will retain for appreciable periods a unity sufficient to permit a definite population to be identified with it. The horizontal movement of the water at a depth of 40 meters should be fairly representative of the layers in which the zoöplankton chiefly occur within the Gulf. At this depth the water is unobstructed in its movements by any considerabe shoals. We have reproduced the dynamic contours at a depth of 40 meters in a recent paper (Redfield and Beale, 1940, Fig. 10). The surface circulation does not differ in important detail from the charts representing conditions at 40 meters. It shows a somewhat closer correlation with certain features of the plankton distribution. Charts showing the gradient currents at the surface have consequently been employed in preparing Fig. 6.

Additional evidence of the character of the circulation, and particularly of its influence in actually transporting a pelagic population, is provided by the distribution of *Limacina retroversa* in the Gulf during the period of this survey (Redfield, 1939). These organisms appeared en masse in the Browns sector in December and their drift was followed as they spread across the Gulf. In four months they had arrived in numbers in the western basin, having spread along the northern margin of the Gulf in the course taken by the receding center of the zoöplankton population (Fig. 7).

The Relation of Population Distribution to the Hydrography of the Gulf

The following theory is proposed to account for the seasonal fluctuation of the population of zoöplankton. The superficial current, or nontidal drift, consists of a great cyclonic eddy. The eddy is augmented by the inflow of water on the eastern side from over the Nova Scotian Banks. The inflow is compensated for by the escape of water to the south and east across the end of Georges Bank. The relative volumes of inflow and outflow vary from season to season and year to year. In the winter and early spring the inflow is sufficiently great to replace a considerable part of the eddy with water new to the Gulf. This "new" water is relatively barren and does not develop a more considerable population until conditions become favorable for growth and reproduction in the spring, by which time it has extended over the entire northern half of the eddy. Meanwhile an equal part of the older water, which had been in the Gulf during the preceding summer, escapes from the Gulf. The remainder occupies the southern half of the eddy. This water supports a rich population grown up during the previous summer and only moderately diminished by the conditions of the winter. In spring and summer the inflow and outflow diminish and the southern half of the eddy carries an increasing quantity of water northeasterly toward the Bay of Fundy, with the result that this water enters a second circuit of the Gulf, carrying with it a large population which enriches the northern half of the eddy during the late summer and fall. This region may also be enriched by an inflow of water from the Nova Scotia banks which carries a considerable population at some seasons.

The adequacy of this theory is demonstrated in Fig. 6, in which contours representing the areas of relative abundance recorded in Figs. 1 and 2 are transposed upon diagrams of the dynamic gradients in the surface waters. The general distribution of these contours was established without reference to the current diagrams.

In September, 1933, the center of population lay in relatively quiescent water along the northern margin of the Gulf and extended southwestward to occupy a secondary eddy over the western basin. A more scanty population occupied the Cultivator sector. Over the Nova Scotian Banks a condition of slack water existed with no evidence of indraft except along the eastern margin of the Eastern Channel. This water was scantily populated. A marked eddy occupies the Eastern Channel with its offshore component lying on the western side. This includes a tongue of richly populated water in which much plankton is being carried out of the Gulf. During this period the population is being impoverished by this outdraft.

By December a strong indraft of water over the Nova Scotian Banks has commenced carrying into the Gulf water containing a scanty population. This water contains abundant *Limacina* which occupy the eastern area in which catches of less than 25 cc. of plankton occurred. (Figure 7.) Compensatory movements must be expelling richly populated waters over Georges Bank.

By March scantily populated water has extended along the entire northern margin of the Gulf, carrying with it the population of *Limacina*. Considerable volumes of zoöplankton were then taken only in the southwestern quarter where relatively slack water is found. A small eddy occupies the western basin and in it *Limacina* mingle with considerable remnants of the copepod population. In the eastern half of the Gulf the major eddy is well marked. Along its eastern side water still enters the Gulf scantily populated with copepods and now containing very few *Limacina*. Its western arm is carrying many *Limacina* out to sea.

In April the character of the circulation changes abruptly from a loop to a closed eddy. Invasion of water from offshore has come practically to an end and considerable numbers of copepods which occur at the mouth of the eastern channel have no opportunity of entering the Gulf. In the west a small concentration of copepods persists near the South Channel. The *Limacina* population is now centered over the western basin, but considerable numbers appear to have spread eastward along the southern and eastern arcs of the great eddy.

Up to this time the movements of water and of the populations of copepods and *Limacina* appear to be perfectly correlated. There can be no doubt that the inflow of barren water from the east has displaced a large part of the copepod population from the northern and eastern part of the Gulf, forcing it out to sea over the eastern end of Georges Bank.

In May and June the loop-like character of the circulation reëstablishes itself, but a considerable eddy persists in the center of the loop. Reproduction now increases the population everywhere. The copepod population occurs in greatest numbers in the slack water of the western basin. From there a rich band extends along an east-flowing current out to sea over the Eastern Channel. A part of this eastward extension has evidently been caught in the recurrent eddy and carried northward toward Mount Desert. Richly populated water found in the North Channel appears to be moving out of the Gulf. The only water entering the Gulf at this time lies along the east side of the Eastern Channel and appears to be scantily populated. It seems improbable that a considerable population is being recruited at this time. The increasing numbers

observed in the eastern region apparently come from the southern and western region.

The distribution of *Limacina* in May–June agrees with this interpretation of the water movements. The population of large specimens, which had wintered in the Gulf, extends eastward along the southern side of the Gulf, and northward along the eastern side of the eddy. The small specimens, new to the Gulf, lie along the inflow and about the center of the eddy. Others follow the eastern arm of the inflow which recurves along the Nova Scotia shore.

By September, 1934 conditions have reëstablished themselves much as they were a year before. The southern half of the Gulf appears to be occupied by the more scanty population, presumably derived from the barren water which lay to the north in the spring. This is trapped in a dead water. A large population has grown up in the eddy which forms each summer in the northeast quarter and well-marked currents exist to carry this population to the southeast. The current flow into the Gulf is stronger than the year before and appears to bear an abundant population from offshore into the eastern side of the Gulf. This is the only indication that the copepod population is enriched by exchanges with offshore waters in the course of the year.

The small numbers of *Limacina* which occupied the Gulf in September, 1934 occurred in greatest numbers along the course of the inflowing water.

It is unfortunate that a more complete survey was not made between June and September. The events which are least clear are those leading to the development of the exceptional populations in the Mount Desert region in late summer. It is not certain that these may not have been recruited from offshore during the summer. The circulation calculated for May–June would appear to transport the richer water then found in the south out to sea more effectively than toward the northeast. Possibly the rich population extending northward toward Mount Desert arrived there before the loop-like eddy reestablished itself. There are, however, several considerations which support the view that the population of the northeastern sectors is recruited from the southern part of the Gulf in early summer.

Fish (1936) records the invasion of the coastal waters of Maine by *Calanus finmarchicus* larvae in June which he assigns to a "western stock." These he believes to be absent from the eastern half of the outer Gulf earlier in the season, and to have drifted in toward the Maine coast from the southwest. The larvae of this stock greatly outnumber those of an "eastern stock" which entered the Gulf from the Scotian Banks in April.

Drift-bottle observations indicate an actual movement of water from the southern toward the northeastern quarter of the Gulf in summer (Bigelow, 1927). There can be no doubt that the surface water does move in this direction, dynamic calculations notwithstanding.

It is possible that lateral mixing along surfaces of equal density may permit rather extensive exchanges of water across gradient currents (Iselin, 1939). In particular, according to a principle developed by Parr (1936), stratification in turbulent waters leads to increased lateral mixing. Thermal stratification in the Gulf of Maine was well developed in May, 1934, and its onset may have facilitated the transfer of well-populated waters across the eddy. It is noteworthy that during May a considerable intermingling of *Limacina* with water rich in copepods occurred along the southern side of the Gulf. It is also noteworthy that the distribution of *Limacina* became much more homogeneous in May than it had been earlier. This was true also of the plankton population as a whole, as Fig. 4 shows. Lateral mixing deserves more study by biologists, as Iselin has pointed out, for it may well be an important factor in preventing local breeding stocks from being swept out of embayments by directional currents.

In summary, the hydrographical evidence appears to support the view that the scanty population of the eastern sectors in midwinter is due to their occupancy by the barren water, which appears in the Browns Bank sector in December and can be traced until it enters the Massachusetts sector by May. The eastern and northern sectors thus receive an influx of relatively barren water in midwinter when the climate is unfavorable for further growth of the population. In the early summer, on the other hand, water drifts from the southern part of the Gulf into the northeastern sectors. This water is about to commence its second circuit of the Gulf and carries with it a population which has already grown to some magnitude in the sectors from Massachusetts to Georges Bank by the end of May.

In contrast to this, the sectors of the western basin receive in winter water which had acquired an abundant population in the Mount Desert and Seguin sectors during the fall. Although there is some destruction of the organisms at this season, it is not sufficient to reduce the numbers greatly. With the coming of spring, this water moves on to be replaced by the barren water found in the northern sectors during the winter. But as this water warms, its population grows and rapidly comes to equal that of the water it replaces. The uniformity of the population in the western basin is due to the fact that a rich fauna arrives there coincident with unfavorable conditions in winter, and a scanty fauna comes to occupy the region during the period most favorable for growth.

The Average Monthly Catch

The monthly catch obtained by averaging the mean values for all sectors during each cruise is given in Fig. 8. The average monthly catch remained constant at about 40 cc. per square meter from June to December, 1933. The values fall markedly from January through April. This is undoubtedly due to the destruction of the population by winter cold. At this period, however, large quantities of water poor in population are entering the Gulf and an equivalent quantity bearing a richer population is leaving, thus accounting for much of the loss.

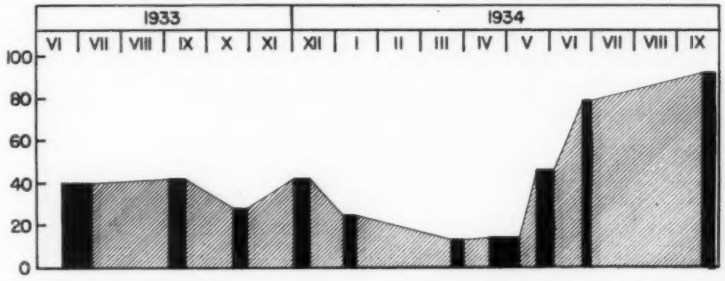

Fig. 8. Average catch for entire Gulf during the period of survey. Ordinate: cubic centimeters of zoöplankton per square meter of sea surface. The black bars indicate the actual period occupied by each cruise.

In May the average population begins to increase suddenly and its growth continues at a diminishing rate until the last observations in September, 1934. The population as a whole is then twice as great as that encountered during the preceding year,—the average haul being 90 cc. per square meter.[2]

[2] Bigelow found that the zoöplankton was at its lowest ebb in late February and the first half of March in 1920. At this time his catches varied from 75 to 25 cc. per square meter measured wet (equivalent to 15 to 5 cc. measured "dry"). Our catches in March averaged 13 cc. and for April 8 cc. per square meter measured "dry." The 1934 year appeared to be a late year and was initiated by an unusually cold winter. The April observations are perhaps misleading since collections were not made in the parts of the Georges and Cultivator sectors where the highest population was expected. Bigelow considered 100 cc. wet (20 cc. or more measured dry) to be representative of the Gulf in midsummer, a value much smaller than our average of 40 cc. in 1933 and 80 cc. in 1934. His largest catch of 425 cc. (85 cc. "dry"), made in September, 1915, does not exceed the average value obtained in September, 1934 for all sectors. It must be remembered that he employed a different method of measuring his catch and nets which doubtless differed from ours in efficiency.

The Total Annual Production and Exchange

The average catch throughout the period of the survey in each sector, —obtained by averaging the mean figures obtained at the time of each cruise—is given in Fig. 9.[3] It is noteworthy that the catch increases progressively as one passes along the course of the water movement from its inflow over the Browns Bank sector to its exit across the end of Georges Bank. The longer the water has been in the Gulf, the greater its population.

The average catch is greatest in the Mount Desert sector and is an exception to the foregoing tendency. We suggest that this is due to the movement of water, which has already completed the circuit of the Gulf and is rich in plankton, from the southern sectors into the Mount Desert sector during the summer. The Mount Desert sector thus virtually occupies a position at the end of the series during the months when its population is greatest.

The average haul for all sectors of the Gulf and at all cruises is about 40 cc. per square meter of "dry" plankton. If the area of the Gulf be taken at 36,000 square miles, this would indicate a total population of about 3.7×10^{12} cc. or some four million tons.[4]

The standing crop does not give a measure of the rate of production of the population, since it reflects merely a balance between rate of growth and death and the gains and losses in the population by movement into or out of the region. It is clear from Fig. 8 that the crop increased by nearly 80 cc. per square meter of surface between May and September. This represents a net gain of some eight million tons.

The observations make it apparent that the Gulf loses at least one half of its population through the escape of water over Georges Bank and the Eastern Channel each winter. There is no evidence that water enters the Gulf at any time carrying a richer population than that obtaining there at the time, and only in September, 1934 was water found to enter the Gulf in which the copepod population was not distinctly

[3] This method of averaging avoids overweighting the yield of these sectors in which an unusual number of rich hauls were made at a time when the population was particularly large, as would be the case if all catches were simply averaged.

[4] In discussing the productivity of the Gulf of Maine, Bigelow concluded that the population was greatest over a band extending from the Massachusetts coast to Penobscot Bay and the Bay of Fundy. The areas occupied approximately by our Seguin sector and the greater part of the Cultivator and Georges Bank sectors were considered barren. His conclusion that the southern sectors are barren certainly rests on insufficient evidence, since only two hauls are recorded. Our richest haul was obtained over the southeastern deep in June, 1934 and the Cultivator and Georges sectors are the most populous sectors excepting Mount Desert throughout the year as indicated. Our observations agree that the Seguin sector is relatively unproductive. Its best season was the fall of the year, a time when Bigelow made relatively few cruises. Several of our richest hauls were made off Seguin in September, 1934.

scanty. Unless further observations during the summer should prove the contrary, it may be concluded that the Gulf is a region of production for the calanoid community which supplies immigrants to the southern banks in quantity, but receives relatively unimportant recruitments from the regions to the eastward.

The Growth of the Population in the Moving Mass of Water

If the interpretation which we put on the data is correct, it is certain that observations made at a geographically fixed point, or standard station, tell little about the fluctuations of any unit of the population. We record simply a series of events distributed in space as they drift past in the course of time. The conditions we observe today are not determined by the events we observed yesterday. The curves of population growth presented in Fig. 5 are grossly misleading if they are interpreted to represent the history of any biologically continuous unit.

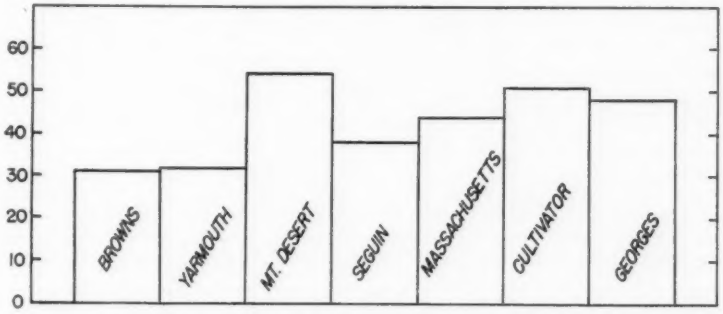

Fig. 9. The average catch in each sector during the period June, 1933 to September, 1934. Ordinate: cubic centimeters of zoöplankton per square meter sea surface.

By taking account of the rate of drift of the water, it is possible to select appropriate stations to show the growth of population in a unit volume of water as it is carried about the Gulf of Maine eddy at the apparent rate of its non-tidal drift. While the result is both an abstraction and an approximation, it probably indicates the true history of events better than the usual curves of population fluctuation. Figure 10 represents the apparent growth of the population of a unit mass of water entering the Gulf in December and recognized by the presence of *Limacina*. As the season advances it is carried across the northern sectors, arriving by May in the offing of Cape Ann. During this period the population decreases by about one-third, but in May as it crosses the western basin rapid growth occurs with the result that in midsummer

it has reached the value of 50 cc. per square meter, greater than the yearly average for the Gulf as a whole. The unit may now drift out of the Gulf across the end of Georges Bank in late summer or it may drift northeasterly into the Yarmouth sector to commence a second circuit of the Gulf. Its numbers grow meanwhile to over 100 cc. per square meter. After January 1, high mortality again reduces the numbers to about one-third or to 30 cc. per square meter, as it crosses the Massachusetts sector in March. In May growth recommences and by

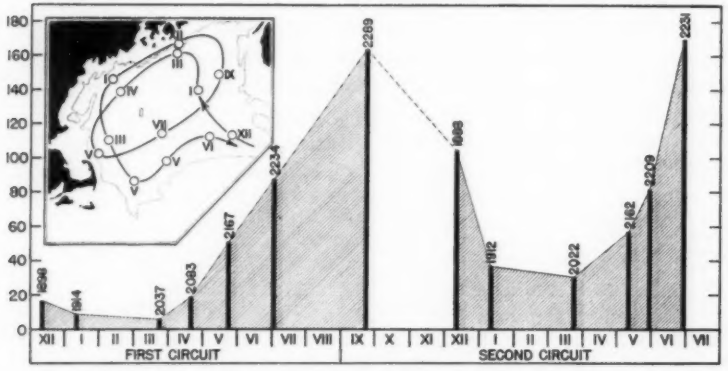

FIG. 10. The growth of population in a mass of water assumed to move along course indicated in inset. Ordinate: volume of zoöplankton in cubic centimeters per square meter sea surface; abscissa, time in months. The black bars indicate the volumes caught at the selected stations. The positions of these stations and the month of collection are indicated on the inserted chart.

midsummer it reaches the Georges sector, having attained the record volume of 170 cc. per square meter. Its history may be terminated by supposing it to be carried out of the Gulf at this time.

Figure 10 is presented because it illustrates the possibility of taking account of the current system in an ecological analysis. It may be pointed out that the life history of any individual species might be treated in a similar way.

ANNUAL FLUCTUATIONS IN POPULATION

The average catch at all stations was more than twice as great during the summer of 1934 as during the same period in 1933. Since the hauls were numerous, widely spaced, and all made with the same technique, there can be little doubt of the significance of this observation. The difference is the more striking in that an unusually severe winter preceded the richer year.

The suggested theory offers a tentative explanation of such fluctuations. The poverty of the water in the northern half of the Gulf in winter seems due to the introduction of a large volume of relatively barren water from the Scotian Banks. It is suggested that more of this water enters in some years than in others, and that the population of the Gulf is impoverished in proportion to the magnitude of this inflow. The longer water remains in the Gulf, before being replaced by new water, the richer its population becomes.

A number of facts support this suggestion. It is well known that the magnitude of the inflow varies from year to year. The inflow during the winter of 1934 seems to have been of shorter duration than usual, having been completely terminated by a strong movement of water from the Gulf over the North Channel and adjoining banks in May. The movement was apparently underway in the latter part of April, as the current diagrams show (Fig. 6). Drift bottles set out by Dr. Herrington off Cape Sable at that time were recovered to the eastward. In contrast to this, Bigelow observed the invasion of the Gulf by Nova Scotian water to continue until May or June and to result in a cooling of the eastern part of the Gulf long after the other parts had commenced to warm. He speaks of the invasion as a phenomenon of spring, whereas in 1934 it terminated before the end of winter.

It is also possible that differences in the circulation such as those observed in September, 1933 and 1934 may cause the population of the Gulf to be augmented from external sources to a different degree each season.

Since the water flowing into the Gulf over the Nova Scotian Banks is less saline than that occurring at like depth within the basins, a small influx of this water should be followed by a summer of relatively high salinity. The superficial water of the Gulf was exceptionally salt in 1934. It appears to have been as salt or salter than in 1915, the most saline year recorded by Bigelow. In 1933, on the other hand, the water was quite as fresh as in 1914 and 1916, the least saline of those he recorded. A correlation between salinity, productivity, and annual inflow over the Scotian Banks is strongly suggested. Systematic annual observations would serve to test this relation, and should it prove general, might lead to an understanding of the yearly fluctuations in commercial fisheries.

The Distribution of Petrels and Mackerel

The zoöplankton supplies food for various predators. Their distribution may be expected to be influenced secondarily by the hydrographic factors which determine the abundance of the calanoid community.

Petrels

These birds appear to feed upon zoöplankton or their products. They pick up whatever scraps of organic matter they can find, gathering about fishing vessels, following ships, and feeding about the carcasses of dead whales and seals. There is a general belief that they pick up droplets of oil from the surface of the water, and their stomachs frequently contain an oily fluid which they eject when captured. It seems

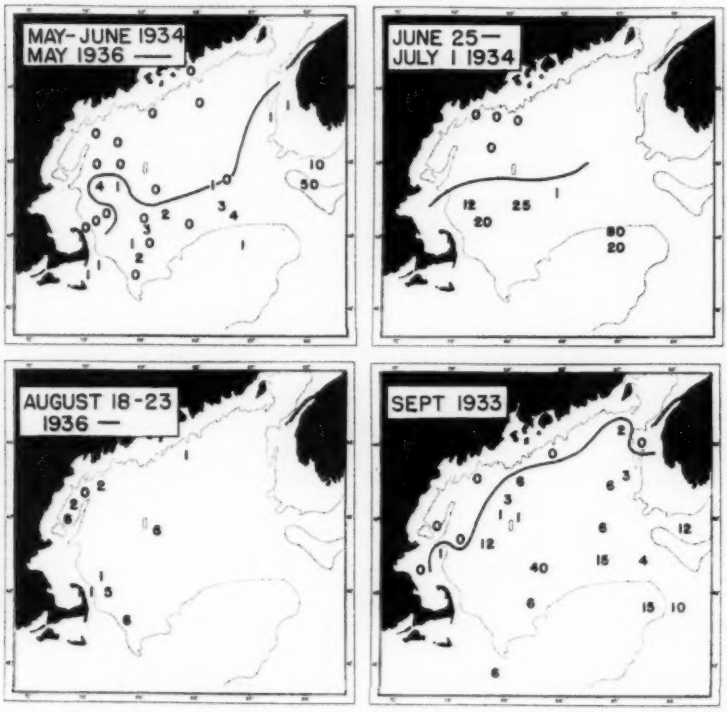

FIG. 11. Numbers of petrels observed in different parts of the Gulf of Maine during cruises at various times of year.

more probable that this is derived directly from their food. Wilson (1907) states that the food of the Wilson's petrel, which he observed in the antarctic, consists of minute crustaceans. The natural food of the Leach's petrel, according to Bent (1922, p. 143) " includes shrimps and other small crustaceans, floating mollusks, perhaps small fishes occa-

sionally, and probably many other forms of minute marine animals which are found swimming on the surface or in floating masses of seaweed."

Wilson's petrel is the common petrel of the Gulf of Maine. Leach's petrel, though a breeder along the coast of Maine, is much scarcer. Not more than one petrel in twenty or thirty observed at sea is of this species. Wilson's petrel breeds in the south Atlantic during December, January and February and does not reach the Gulf of Maine until May. None were observed during the cruise of April 19–23, 1934. In cruises in May, 1934 and 1936 petrels were observed in small numbers in the southern and eastern regions, where at that season the largest zoöplankton hauls were taken, but were absent from the northern sectors which at the time were occupied by relatively barren water (Fig. 11). During

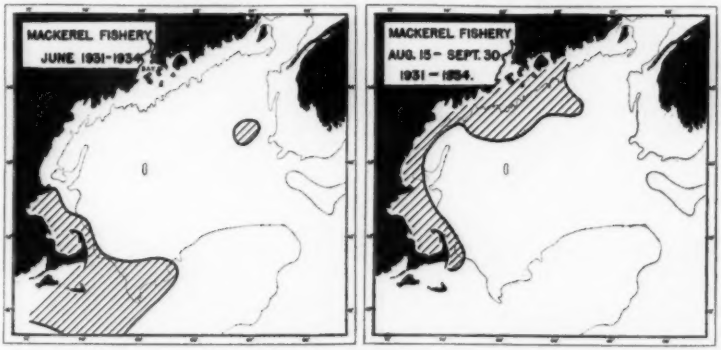

FIG. 12. The areas occupied by the mackerel fishery during the early and late summer 1931–1934.

a cruise from June 25 to July 1, 1934, petrels were present in larger numbers in the southwestern quarter, but were not observed in the Seguin sector. On August 18 to 23, 1936, however, petrels were observed regularly at stations made in the northern side of the Gulf. In September, 1933 petrels were observed everywhere in the Gulf except at a few coastwise stations along the shore. At this time all sectors supported an abundant population of zoöplankton.

It appears that when Wilson's petrel first arrives in early summer it remains confined to those sectors of the Gulf which then support the richest plankton.

Mackerel

In the Gulf of Maine these fish have long been known to feed on calanoid copepods and are known to eat various other crustaceans which

compose the bulk of the zoöplankton (Bigelow, 1926, p. 102). It is not surprising in consequence to find a correlation between the seasonal distribution of this fishery and of the local abundance of zoöplankton.

Dr. Settee has kindly placed at my disposal the very complete records made by the U. S. Bureau of Fisheries showing the places in which mackerel have been taken during recent years. The mackerel fishery begins in New England waters with the arrival of the fish south of Cape Cod in May and June. The fish are first taken along the shores of the Gulf of Maine in June, chiefly within the 100-meter contour from Cape Ann to Cultivator shoal. At this time heavy catches are also made along the east coast of Nova Scotia and northward to Gaspé (Settee and Needler, 1934). The July fishery has much the same distribution, though tending to spread farther east along Georges Bank and also in some years along the western part of the coast of Maine. In August and September a considerable fishery is conducted in the northern side of the Gulf, from Mount Desert westward, and southward as far as Cape Cod (Fig. 12). During these months the fishery in the Bay of Fundy is at its height, 37 per cent of the total catch being made during each of these two months, whereas less than 10 per cent is taken in any one of the preceding or following months.

In interpreting these facts the peculiarities of the fishermen as well as of the fish must be borne in mind. Mackerel are now marketed fresh and are landed chiefly in Boston. The fishermen consequently do not fare farther from this port than is necessary. The takings of mackerel do not reflect accurately the total distribution of the fish, but only their availability to the Boston market. It seems sufficiently clear that in early summer mackerel are available chiefly along the southern shores of the Gulf; that by late summer their abundance has shifted to the northern shores, including the Bay of Fundy. This is the distribution of the maximum of zoöplankton population as well. Whether the mackerel follow the plankton as it drifts around the great eddy, or cut across to meet its advance from the east in the late summer, as the fishermen undoubtedly do, cannot be told.

Thus there appears to be a general correlation between the distribution of the zoöplankton, the occurrence of petrels, and the capture of mackerel.

Summary

1. The seasonal and geographical fluctuations of the abundance of the calanoid community of the Gulf of Maine are described.

2. The shift in the center of abundance is closely correlated with the superficial circulation, deduced from hydrographic observations and the drift of an invading population of *Limacina*.

3. The principal factor influencing the distribution of population density is the inflow of relatively barren water from the Nova Scotian coast in winter.

4. The Gulf appears to be an area from which the calanoid community spreads to other waters, but which receives relatively small recruitments from without its borders.

5. A breeding stock is maintained by the establishment of a recurrent eddy in the late spring.

6. Estimates of annual productivity and seasonal mortality are given.

7. The distribution of petrels and of the mackerel fishery appears to be correlated with the distribution of zoöplankton.

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CHANGES IN THE TISSUE CHLORIDE OF THE CALIFORNIA MUSSEL IN RESPONSE TO HETEROSMOTIC ENVIRONMENTS[1]

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The adult California mussel, *Mytilus californianus* Conrad is a fairly heterosmotic animal, typically marine in its environment, yet potentially euryhaline in a striking degree.

It has been shown (Fox et al., 1936) that this mollusk can live in the laboratory for long periods following sudden and continued immersion in aerated solutions of natural sea salts, varying in Cl concentration[2] from about 0.94 per cent, or approximately half that of natural sea water in the vicinity of La Jolla, to as high as 2.5 per cent, or about 34 per cent above normal values (1.86 per cent being the approximate Cl concentration of sea water at La Jolla).

Sudden exposure to concentrations of sea salts below or above these respective values proved fatal to mussels, but gradual alteration of the water in which they were immersed, over periods of several weeks, left the animals still alive in solutions diluted, on the one hand, to one-third of the normal Cl concentration (0.62 per cent Cl) or concentrated, on the other hand, to nearly twice the normal value (3.50 per cent).

The purpose of this investigation was to determine the chloride concentration in the tissues of the mussel, under the widely differing physiological conditions which must result from continued exposure to concentrations of sea salts not encountered by these animals in nature.

Extensive reviews of both older and more recent work dealing with homoiosmoticity and heterosmoticity of fishes and invertebrates have been presented in papers by Dakin (1935) and by Schlieper (1935).

[1] Contributions from the Scripps Institution of Oceanography New Series No. 117.

[2] Cl concentration refers to the total halide ion concentration as determined in sea water analyses. The same designation, used in the present discussion, refers to the total halide concentration in parts per cent of both water and tissues, since the amounts of both Br and I are relatively very small. Concentration of "chloride" refers hereinafter to that of halide ions.

Other useful discussions of the subject are given by Adolph (1930) and by Baldwin (1937).

Experimental Methods

Diluted sea water solutions were prepared by adding distilled water to sea water, while relatively concentrated solutions were obtained by the partial evaporation of ordinary sea water without precipitating any salts.

Experimental solutions were contained in glass battery jars, each of 2½ gallon capacity. To avoid any injurious effects which might result from overcrowding, the predetermined ratio of at least one liter of water per animal was consistently adopted as a minimum. Mussels of an average length of 10 cm. (varying between 9 and 11 cm.) were employed in the great majority of experiments, although no biochemical or physiological differences of direct bearing upon the work were recognized in larger animals (12 cm.) or in somewhat smaller ones (7 to 8 cm.)

Experiments were carried out at room temperatures which varied between the approximate limits of 19° and 22° C. All solutions containing mussels were aerated continuously.

No attempts were made to adjust and maintain the pH of the various solutions, since it was found that mussels lived in piped sea water within the range of pH values encountered in the experiments. Figures representing numerous pH measurements of environmental solutions will serve as examples and are tabulated below. These measurements were made with a Beckman glass-electrode pH meter through the kindness of Mr. J. C. Hindman.

Solution	pH
(1) Sea water of normal salinity from running supply in tank containing mussels; unaerated	8.63
(2) The same; aerated	8.43
(3) The same, drained from mantle-cavity of mussel; unaerated	7.51
(4) Sea water of normal salinity from stationary supply in large jar containing mussels; unaerated	7.50
(5) Sea water, initially of normal salinity, diluted to maximum degree used in experiments, i.e. to one-third of original concentration (ca 0.62 per cent Cl); aerated	8.52
(6) Sea water, initially of normal salinity, concentrated to maximum degree used in experiments, i.e. to 54 per cent of original volume (ca 3.50 per cent Cl); aerated	8.32

Since the mussels were therefore presumed not to have been exposed to unfavorable conditions of acidity or alkalinity during the course of the experiments, it was concluded that extensive changes in the concentration of dissolved salts themselves constituted the limiting factors to life in the environment. The possibility of disturbances in "salt balance" (in the physiological sense), of experimental solutions was considered. Because calcium was believed to be the chief element prone to be precipitated as the carbonate from moderately concentrated sea water, analyses were made for dissolved calcium in sea water samples concentrated by boiling. Normally present in amounts close to 0.42 gram per liter, the dissolved calcium in a solution boiled down without precipitation to 54 per cent of its original volume (number 6 in above table) was present in the nearly theoretical amount of 0.80 gram per liter. Since this solution was the most concentrated used in any of the experiments, it was concluded that mussels were at no time exposed to solutions "unbalanced" with reference to calcium.

Experimental animals were introduced into the various solutions of altered salinity after first propping the valves apart by a few millimeters with smooth glass plugs and draining the gill chamber of sea water. Animals immersed under these conditions in sea-salt solutions varying between the approximate limits of 0.95 per cent and 2.73 per cent Cl usually relaxed their hold on the glass rod in a short time, parted their valves in a normal manner, and resumed feeding activities.

The flesh of control and experimental mussels was prepared for chloride analysis in the following way. The mussels were removed from their shells as rapidly as possible and with minimum cutting of tissues, severing only the adductors and small muscles attached to the hinge region of the shells. The flesh was blotted on absorbent paper to remove most of the adhering sea water, then rinsed briefly in 95 per cent ethyl alcohol (and reblotted) in order to remove most of the remaining sea water from body surfaces, constrict the gill capillaries, wash out sea water expressed therefrom, and coagulate the cut surfaces to allay excessive bleeding and subsequent losses of chloride-containing body fluids. The consistent adoption of this procedure resulted in a series of checks which were quite close in normal control animals, in spite of the fact that the work was done on the wet weight basis.

For chloride analysis, the method of Sunderman and Williams (1931, 1933) was followed, digesting the whole tissues in chloride-free KOH, followed by further treatment of aliquot portions with concentrated HNO_3 in the presence of an excess of dilute $AgNO_3$ solution. The excess of Ag ion was finally titrated with standard NaCNS solution in

TABLE I

Chloride concentrations in wet tissues of mussels, showing slight variations with sex, maturity and season.

Date	No.	Sex	Weight of flesh	Percentage Cl		
			grams			
8–11–37	1	♂ (much sperm)	22.88	0.99		Summer.
8–13–37	2	♂ " "	18.70	0.83		
8–13–37	3	♀ (many eggs)	14.85	0.89		
"	4	♂ (much sperm)	16.57	0.89		
"	5	♀ (many eggs)	16.50	0.82		
"	6	♀ " "	13.23	0.805	av. % Cl in whole ripe ♂ = 0.91	
"	7	♂ (much sperm)	17.59	0.83	av. % Cl in whole ripe ♀ = 0.87	
8–17–37	8	♂ (mature)	12.51	1.00	Grand av. = 0.90	
"	9	♂ "	10.025	0.965		
"	10	♀ "	8.52	1.065		
"	11	♂ "	11.50	0.81		
"	12	♀ "	10.61	0.77		
"	13	♂ (immature)	6.56	0.99		
	Gonads only	♂	16.95	0.90		
	" "	♀	15.68	0.61		
	" "	♂	15.03	0.945	av. % Cl in ripe ♂ gonad = 0.89	
	" "	♀	13.86	0.58	av. % Cl in ripe ♀ gonad = 0.57	
	" "	♂	10.24	0.82		
	" "	♀	13.61	0.52		
12–13–37	1	? (immature)	6.41	0.97		Winter.
"	2	♂ "	6.26	0.98		
"	3	? "	7.98	0.96	av. % Cl in immature animals = 0.98	
"	4	? "	8.19	0.98		
"	5	♂ "	5.96	1.005		
"	6	♂ "	5.08	0.995		
1–31–38	1	♀ (many eggs)	12.41	1.00		
"	2	♀ (spawned)	9.84	0.91		
"	3	♂ (much sperm)	16.37	0.96		
"	4	♂ " "	18.16	0.97	av. % Cl of sperm—cont'g ♂ = 0.97	
"	5	♀ (many eggs)	12.08	0.93	av. % Cl of egg—cont'g ♀ = 0.93	
"	6	♂ (much sperm)	15.69	1.01	av. % Cl of all ♀ = 0.945	
"	7	♀ (many eggs)	17.94	0.905	av. % Cl of all animals = 0.96	
"	8	♂ (much sperm)	16.49	0.945		
"	9	♀ (many eggs)	21.20	0.90		
"	10	♀ (spawned)	12.38	1.03		

the presence of ferric alum according to the well-known method of Volhard. The Volhard method was also employed for the analysis of chloride in sea-water solutions. Our experiments showed that the step involving preliminary digestion of tissues by KOH gave consistently higher values for chloride than did the ordinary "open Carius" determinations (digestion with excess concentrated HNO_3 and $AgNO_3$) employed by other workers. Sunderman and Williams (1933) report incomplete recovery of chloride when the preliminary alkaline digestion is omitted, and assign the low chloride values to interference by fatty substances.

Preliminary Analytical Survey of Normal Animals

Because parts of this research were conducted in different seasons of the year, i.e. especially in the summer and fall of 1937 and the winter of 1937–1938, it seemed desirable to compare chloride analyses of normal animals taken during August with those of animals comparable in size and weight taken in December and January. Also, because it was impossible to differentiate the sexes without sacrificing the animals' lives, and since it was conceivable that biochemical differences in sex might be reflected in the chloride content of the tissues, attention was given to the sex and relative degree of maturity throughout the same group of animals.

The data of Table I reveal that differences between the chloride content of whole bodies of summer animals and those of winter animals are of relatively small order, showing a departure of only + 0.06 per cent in the grand average, in favor of the winter animals; this difference is the same in direction and extent whether one compares ripe winter with ripe summer males, or ripe winter with ripe summer females.

Sexually immature animals exceeded in chloride content the grand average (0.93 per cent) of the combined values of mature animals of both seasons by the small departure of 0.05 per cent.

Because the demonstrated seasonal and sexual differences in chloride content fell well within the departures recorded between individual analyses, they were not regarded as significant in the experimental results. The sexual difference in chloride content appeared to be a real one, although relatively small, and for the purposes of this work, insignificant. It was doubtless due to the fact that the relatively heavy ripe ovary contained only about two-thirds as much chloride as did the male gonad.

Changes in Tissue Chloride Following Sudden Immersion in Heterosmotic Solutions of Sea Salts within the Tolerated Physiological Range of Concentrations

Reference to Figs. 1 and 2 brings out some rather consistent general facts: While mussels in nature show close agreement among one another

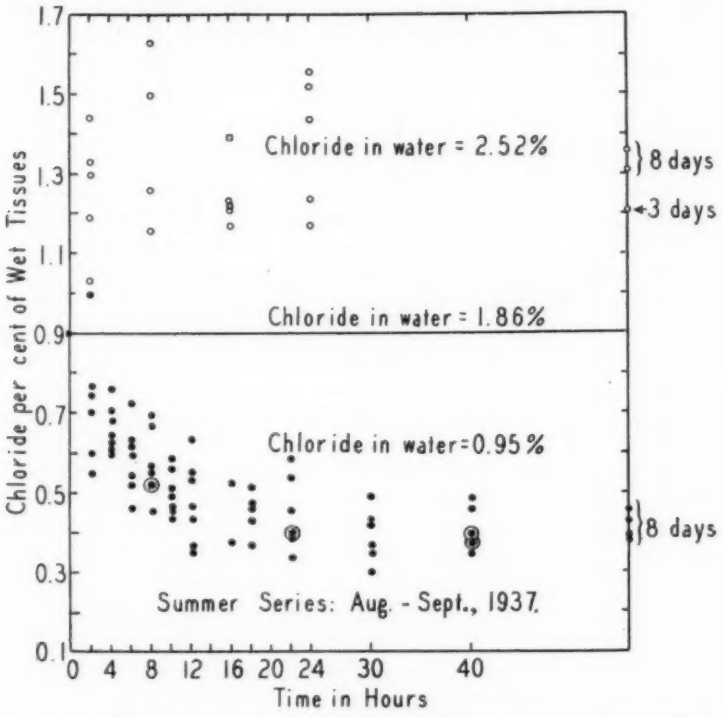

FIG. 1. Chloride analyses of whole mussel tissues following immersion of living animals for increasing time-intervals in hypotonic and hypertonic sea water within the limits of the tolerated range. Each point represents the analysis of a single animal; where two or more analytical values were identical, this is shown by concentric circles.

in chloride content, wide individual variations are manifest in the rate of change in tissue chloride concentrations in both kinds of new environment. This is particularly striking in the early hours following immersion. Since mussels have been demonstrated to survive for long periods at either the hypotonic or the hypertonic concentrations here

employed, and to eventually attain, in each group, respective chloride levels in close individual agreement, the obvious differences in the early hours are clearly due to individual variations in rate of water and salt interchange.

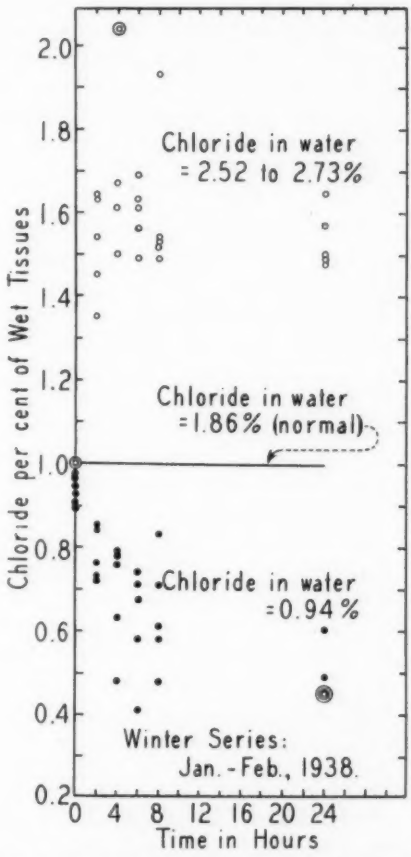

FIG. 2. Further chloride analyses, as in Fig. 1.
See Fig. 1 for meaning of points and concentric circles.

While four out of five animals which remained for the whole 24-hour period in water containing 2.52 to 2.73 per cent Cl (Fig. 2) were filtering water, feeding, and voiding feces and thus appeared normal, none of the animals in the water containing 2.78 per cent Cl (Fig. 3) appeared

normal even after 30 hours; they maintained a grip on the inserted glass props, failed to filter water, and gave off much mucus, thus giving several signs of physiological disturbance. A later lot, however, exposed to a solution of 2.80 per cent Cl (Table IV) did not show any effects of injury after 25 hours. These facts are taken as evidence that Cl values between 2.70 per cent and 2.80 per cent are close to the threshold of hypertonicity at which the mussels can withstand sudden immersion.

Figure 4, to which reference will be made below, reveals the close fit to a straight line *between water chlorinities tolerated by mussels after sudden immersion.* Attention is directed, for the moment, only to the

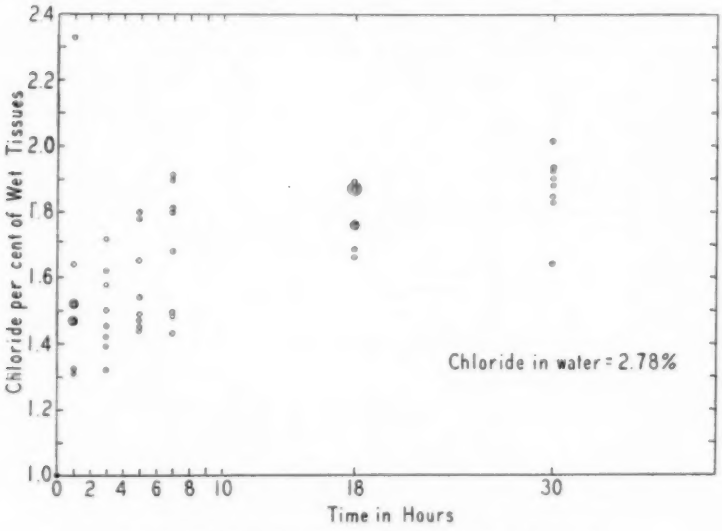

FIG. 3. Further chloride analyses, as in Figs. 1 and 2. See Fig. 1 for meaning of points and concentric circles.

portion of the graph lying between the ordinate values of 0.95 per cent Cl and 2.8 per cent Cl, illustrating the data shown in Figs. 1 and 2, and those of Table IV, when all average values of internal chloride concentrations attained by mussels in 24 to 40 hours are plotted against chloride concentrations of external water. The ratio between grams Cl per 100 grams wet mussel tissue and grams Cl per 100 ml. sea salt solution has an average value of 1 : 1.89.

The blackened point on Fig. 4 indicating the average chloride concentration in the tissues of 8 mussels maintained for 30 hours in water containing 2.78 per cent Cl was obtained from Fig. 3. While this

average value lies in the vicinity of the curve, its departure is doubtless due to the fact that the animals were in poor condition (from an unknown cause), and so failed to maintain the ratio between internal and external chloride exhibited by other lots of animals at this concentration.

Specimens placed, with valves propped apart, in distilled water for 15- or 16-hour periods underwent a drop in tissue chloride to values of 0.226 per cent or even 0.112 per cent. Under these conditions animals, although not quite dead, were definitely moribund, and failed to recover when placed in running sea water.

Tolerance by the Mussel of Gradually Altered Concentrations of Sea Salts

While the immediately preceding experiments and earlier investigations seemed to define fairly well the limits of hypo- and hypertonic solutions withstood by mussels following *sudden* immersion, there were grounds for believing that *gradual* changes in the salt concentration in the animals' environment might not bring about signs of injury until greater extremes in both directions were reached.

In order to investigate this question, and to determine if possible the extremes of tissue chloride lost or gained by surviving animals, the following experiments were conducted. Two sets of mussels in separate jars of normal sea water, continuously aerated, were subjected to a gradual change in the chloride concentration of the water, one jar being diluted, the other concentrated daily by slow steps over a period of about six weeks. This gradual change was brought about in the following manner. From one jar containing about eight liters of sea water (seven mussels) some 250 ml. were withdrawn, discarded and replaced by an equal quantity of distilled water daily; from the other jar (eight mussels) the same volume of water was discarded and replaced by an equal quantity of water containing sea salts concentrated by about four-fold (i.e. Cl concentration of 7.725 per cent). These changes brought about a gradual drop in Cl from 1.86 to 0.625 per cent in the first, and a gradual rise from the same initial value to 3.48 per cent in the second solution, over the experimental period. Under these special conditions the mussels survived exposure to previously unexpected concentrations at both extremes.

Table II shows the results of chloride analyses, and a restoration of experimental animals to normal chloride levels after exposure to the gradually altered solutions during five and six-week periods.

It is assumed from the foregoing experiments that it has been possible to ascertain the approximate thresholds of tolerance of the mussel toward the osmotic effects of both hypotonic and hypertonic solutions of

TABLE II

Limits of tolerated changes in tissue chloride level.

Date 1937	Approximate time elapsed	Cl in hypotonic water	Cl in mussels	Cl in hypertonic water	Cl in mussels
		per cent		*per cent*	
Dec. 13, 14	5 weeks	0.84	3 animals: 0.41% 0.40 0.33 Average: 0.38%	3.09	5 animals: 2.08% 1.82 1.77 1.79 1.72 Average: 1.84%
Dec. 15	5 weeks	0.84	1 animal from same group, placed in running sea water 6 days, and analyzed: Cl = 1.1%	3.09	1 animal from same group, placed in running sea water 6 days, and analyzed: Cl = 0.935%
Dec. 20	6 weeks	0.625	0.26% (sluggish)	—	—
Dec. 21	6 weeks	0.625	0.53% (moribund) Remaining animal in this group placed in running sea water 13 days, and analyzed: Cl = 0.97%	—	—
Dec. 22	6 weeks	—	—	3.48	2 remaining animals (becoming sluggish) 2.19% 2.32 Average: 2.25%

sea salts. It seemed hardly likely, however, that individuals kept in solutions of concentrations below 0.94 per cent Cl or above 2.8 per cent Cl would survive indefinitely, since the flesh of animals in solutions beyond these respective concentrations appeared rather thin, sometimes even emaciated. Furthermore, they failed, in these respective realms of salt concentrations to maintain their constant ratio of tissue chloride to sea water chloride as illustrated in Figure 4. This graph summarizes much of the information reported above and indicates the relationship which exists between the chloride concentration in the surrounding media and that in the tissues of animals immersed therein. Each point

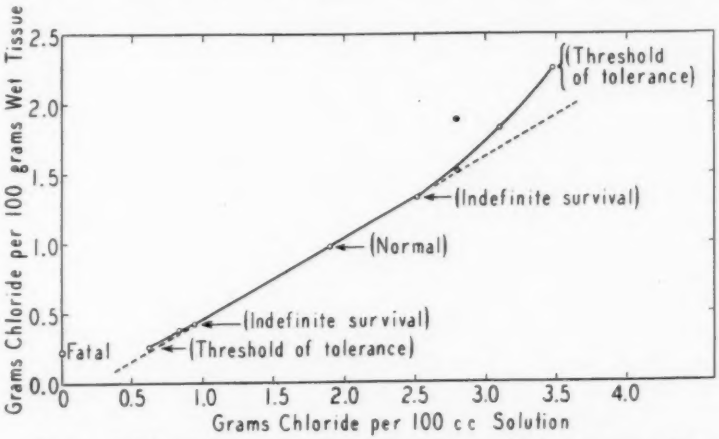

FIG. 4. Relationship between internal and external chloride, in grams Cl per 100 grams tissue vs. grams Cl per 100 ml. water respectively. Points lying between abscissal values of 0.95 and 2.8 represent results obtained after 24 hours or more following *sudden* immersion. Portions of the graph lying below and above these respective values represent the trend taken following gradual alteration of Cl concentration in the water over protracted periods, excepting wherein *distilled* water was used (see text).

represents the average of the analyses of a number of animals immersed in the respective solutions for 24 hours or more, save in the cases of the two lowest points on the hypotonic side, which represent analyses of two respective animals: a single survivor containing 0.26 per cent Cl and the animal which showed the highest Cl value (0.226 per cent) of the group that succumbed to immersion in distilled water (at zero abscissal value). The maintenance of a ratio of 1:1.89 between internal and external chloride is noted in the straight line between the latter concentrations of 0.94 per cent and 2.8 per cent Cl. The projection of this straight line as a dotted line beyond these values shows, when com-

pared with the actual curve, the degree of departure in the animals' maintenance of such a ratio. The final two points at each extremity of the curve represent Cl values attained by mussels after *gradual* changes in Cl concentration of the water to the corresponding values shown on the abscissa. (See Table II.)

Content of Water and Chloride in Various Tissues: Exchanges of both Water and Chloride Ions between Tissue Fluid and Environment

Since some question arose as to whether a considerable part of the observed changes in tissue Cl might be assignable to the mere presence

TABLE III

Water and chloride contents of gills and bodies (minus gills) of normal mussels.

Gills:	Wet weight	11.02 grams
	Dry weight	1.09 "
	H_2O	90.1%
	Cl (wet wt.)	1.32%
	Mols. Cl per liter tissue water	0.413
Bodies minus Gills:	Wet weight	59.25
	Dry weight	7.30
	H_2O	87.7%
	Cl (wet weight)	1.075%
	Mols. Cl per liter tissue water	0.345
Combined Total:	Wet weight	70.27
	Dry weight	8.39
	H_2O	88.06%
	Cl	1.11%
	Mols. Cl per liter tissue water	0.355

of the medium itself in the capillary tubes of the gill structures, analyses were made of water and chloride in (1) the gills and (2) the rest of the tissues en masse, of six mussels taken directly from stock tanks of running sea water. In consistency with the experiment, tissues could not in this case be rinsed in alcohol prior to analysis (hence the slightly elevated Cl values). Table III shows the results of these analyses, and brings out the fact that the gills, as dissected for analysis, possess only a slightly higher moisture content than do the other tissues; furthermore while the chloride content of the gills was about 23 per cent higher than that of the other tissues, their relative proportion of the total wet body weight was only about 15.6 per cent, so that they were responsible

TABLE IV

Water and chloride contents of foot and body tissues (minus foot) of mussels immersed for 25 hours in sea water of three concentrations. N series: normal sea water, Cl = 1.86 per cent; D series: diluted sea water, Cl = 0.97 per cent; C series: concentrated sea water, Cl = 2.80 per cent.

No.	Sex	Wet wt. foot	Wet wt. other tissues	Combined wet wt.	Dry wt. foot	Dry wt. other tissues	Combined dry wt.	Percentage H_2O foot	Percentage H_2O other tissues	Percentage H_2O whole body	Percentage Cl: foot	Percentage Cl: body	Percentage Cl: whole
N 1	? immat.	0.27	10.01	10.28	0.06	1.36	1.42	77.8	86.4	86.2	.765	1.06	1.05
N 2	♂ (?)	0.255	12.83	13.09	0.06	1.72	1.78	76.5	86.6	86.4	.71	1.11	1.10
N 3	♀	0.24	10.12	10.36	0.06	1.44	1.50	75.0	85.8	85.5	.57	1.04	1.03
N 4	♂	0.20	9.72	9.92	0.05	1.36	1.41	75.0	86.0	85.8	—	1.10	—
N 5	♂	0.18	7.94	8.12	0.04	1.02	1.06	77.8	87.15	86.95	.89	1.10	1.09
N 6	♀	0.12	10.80	10.92	0.03	1.35	1.38	75.0	87.5	87.4	.66	1.10	1.10
				av.wt. = 10.45				av. = 76.1	av. = 86.6	av. = 86.4	av. = .72	av. = 1.09	av. = 1.08
D 1	♀	0.23	13.07	13.30	0.04	1.275	1.315	82.6	90.25	90.1	.32	0.51	.505
D 2	♀	0.43	15.03	15.46	0.07	1.51	1.58	83.7	89.95	89.8	.38	.48	.48
D 3	♂	0.365	13.10	13.465	0.065	1.64	1.705	82.2	87.5	87.3	.345	.50	.50
D 4	♂	0.50	15.79	16.29	0.08	1.60	1.68	84.0	89.9	89.7	.36	.51	.505
D 5	♂	0.43	14.45	14.88	0.07	1.65	1.72	83.7	88.6	88.4	.39	.465	.46
D 6	? immat.	0.255	10.78	11.035	0.04	1.11	1.15	84.3	89.7	89.6	.32	.50	.50
				av.wt. = 13.07				av. = 83.4	av. = 89.3	av. = 89.15	av. = .35	av. = .49	av. = .49
C 1	? immat.	0.19	10.96	11.15	0.05	1.58	1.63	73.7	85.6	85.4	1.15	1.60	1.59
C 2	♂	0.105	8.96	9.065	0.025	1.34	1.365	76.2	85.05	84.95	1.715	1.60	1.60
C 3	♀	0.365	8.31	8.675	0.10	1.41	1.51	72.6	83.1	82.6	0.96	1.50	1.48
C 4	♂	0.30	9.27	9.57	0.07	1.37	1.44	76.7	85.2	84.95	1.46	1.62	1.61
C 5	♂	0.405	11.77	12.175	0.10	1.63	1.73	75.3	86.15	85.8	1.15	1.42	1.41
C 6	♀	—	—	12.54	—	—	1.90	—	—	84.85		1.34	1.34
	(Foot atrophied to tiny nub)			av.wt. = 10.53				av. = 74.9	av. = 85.05	av. = 84.75	av. = 1.29	av. = 1.51	av. = 1.505

for bringing the total tissue chloride up from 1.075 per cent to 1.11 per cent, an increase of only about 3.5 per cent. Observed shifts in tissue chloride were therefore not due to environmental solution mechanically suspended in the gills.

The question of whether the change in tissue chloride concentration might be due, at least in part, to the osmotic interchange of major quantities of *water,* with or without the migration of Cl ions as well, was investigated in an experiment involving eighteen animals of the usual range of length (i.e. 92 to 110 mm.), the results of which appear in Table IV. Six mussels were immersed in ordinary sea water, six propped open in hypotonic water ("50 per cent sea water"; 0.97 per cent Cl), and a third set of six propped open in hypertonic water wherein the Cl concentration was 2.80 per cent. After being kept in the respective, constantly aerated solutions for 25 hours, the animals were analyzed. The foot of each was severed with a sharp razor blade and analyzed separately from the other tissues, in order to determine whether

TABLE V

Weight of water and of chloride per unit weight of dry tissue; concentration of chloride per 100 grams of tissue water.

Series	Grams H_2O per gm. dry tissues			Grams Cl per gm. dry flesh			Grams Cl per 100 gm. tissue-water		
	foot	body	whole	foot	body	whole	foot	body	whole
N	3.18	6.46	6.35	0.030	0.081	0.079	0.94	1.25	1.24
D	5.025	8.34	8.22	0.021	0.046	0.045	0.42	0.55	0.55
C	2.98	5.69	5.55	0.051	0.101	0.099	1.72	1.77	1.78

such a relatively compact structure might show any considerable difference in chloride shift as compared with that of softer tissues. The dry weights of all tissues were obtained by keeping them overnight in tared containers placed in an electric oven at 105° C., then re-weighing; chloride was determined in the usual manner. In the table, the N series represent the normal control animals kept in sea water, the D series those placed in the diluted sea water, and the C series those immersed in the more concentrated solutions. Some significant trends are observed. The average water content of foot tissues and of whole bodies shows a consistent increase in the order which would have been expected from a consideration of the osmotic effects of the relative solutions, i.e., $D > N > C$; the relative chloride values of foot tissues or of whole bodies show the opposite order, i.e., $C > N > D$.

The exchanges of both water and chloride ions, under the conditions of the preceding experiment, are brought out in a quantitative way in Table V, showing for each of the three series, the ratios of average

water content and average chloride content, in grams per gram of dry tissue.

Furthermore, by plotting the values for Cl concentration in grams per 100 grams of tissue water, against Cl concentrations in grams per 100 ml. of sea water solutions, we arrive at the nearly linear relationships shown in Fig. 5. The foot, which contains less water, less chloride, and lower concentration of the latter, normally constitutes only about 2 per cent of the total body weight, and exerts no significant influences upon the data collected from analyses of the whole body. Nevertheless,

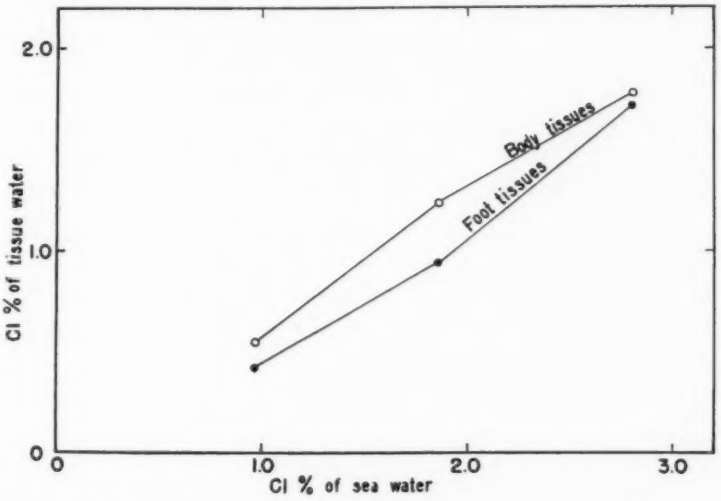

FIG. 5. Relationship between internal chloride concentrations in grams per cent of tissue water and in environmental water respectively. Sea water chlorinities lie within the range of values tolerated by mussels on sudden immersion.

this compact muscular tissue is observed to exchange both water and chloride with the aqueous environment in a manner similar to that of the other tissues.

Under the conditions of the above experiments, the mussel alters, throughout a physiologically wide range, the concentrations of its dissolved salts in such manner as to maintain a rather constant ratio between the Cl concentration of internal and that of external water, this ratio appearing to have an average value of the order of 1 : 1.60. The migration of both chloride and water occurs between the tissues and the solution outside.

Discussion

The ready exchange of water and chloride between the mussels' flesh and the environment may not involve any such profound changes in tissue cells themselves as might seem to be the case. The tissues of some organisms contain major quantities of the total chloride in the interstitial fluid, the cells themselves possessing the ion in very small amounts, and not readily undergoing alterations in water content or chloride concentration. This seems to be especially true in certain muscular tissues of mammals (Eggleton, Eggleton and Hamilton, 1937; Bourdillon, 1937), but some investigators are of the opinion that in other tissues (notably in frogs) considerable portion of the chloride is intracellular (Amberson, Nash, Mulder and Binns, 1938).

Since the completion of the present work, some informative results have been published by Steinbach (1940 *a, b*), who studied the content and distribution of water and electrolytes in the excised retractor muscles of certain marine invertebrates, i.e. the holothurian, *Thione briareus* and a sipunculid worm, *Phascolosoma.*

Steinbach immersed his material for a few hours in solutions containing chloride in concentrations of from 52 milli-equivalents per cent (i.e. full-strength sea-water) to nearly zero, employing isosmotic sucrose, or alternatively $NaNO_3$ solution as the sea water diluent. No hypertonic solutions were used. He obtained linear relationships throughout the experimental range of concentrations employed, the fresh *Thyone* tissue being richer in Cl (20.4 meq. Cl per cent) than that of *Phascolosoma* (9.1 meq. Cl per cent when fresh, and 16 meq. Cl per cent when soaked in sea water), and the former yielding steeper slopes of linear change. The normal water-content was very similar in both species, i.e. 75.9 per cent in *Thyone* and 78 per cent in *Phascolosoma* muscle.

The present writer immersed whole mussels for 24 hours or longer in normal, dilute, and concentrated solutions of natural sea salts within the range successfully tolerated by the animals. Linear relationships were apparent between the range of about 1.74 and 79.1 meq. Cl per cent of environmental water, when dealing with whole bodies, while such data as were collected upon the subsequently amputated foot alone revealed a nearly linear function between environmental Cl concentrations of 27.7 and 79.1 meq. Cl per cent.

It is also of interest to note that the initial concentration of Cl in Steinbach's *Thyone* muscle is virtually identical with that of the muscular foot of *Mytilus,* viz.; 20.4 and 20.3 meq. Cl per cent respectively, and that the water-contents of both are close, viz.: 75.9 per cent and 76.1 per cent. Furthermore, the Cl concentration in half-strength sea water

(Steinbach's fortified with isosmotic solutions of sucrose or $NaNO_3$, mine merely diluted) resulted in a decrease in *Thyone* muscle Cl to a value close to that determined for *Mytilus*-foot, viz.; 12 vs. 9.9 meq. Cl per cent respectively.

Steinbach's experiments led him to conclude that muscular tissues of both *Thyone* and *Phascolosoma* (classed as smooth muscle) contain sodium chloride almost exclusively in the extracellular space, that it is free to equilibrate by simple diffusion with external solutions, and that little if any can penetrate the tissue-cells themselves. He discusses the evidence in support of the conclusion that virtually all chloride is extracellular in the material studied by him, and in striated muscles of frogs and some other vertebrates as well, and that measurement of chloride content of such tissues may be employed as a relative measure of the extracellular space.

The muscular foot of *Mytilus* is observed to show osmotic behavior closely similar to that of the retractor muscles of *Thyone* and *Phascolosoma*, and must be very like the latter tissues in biochemical constitution and function.

The whole body of *Mytilus* shows a similar linear slope as does the foot tissue within the range of environmental Cl concentrations compared, but exhibits consistently higher water and Cl content. Deviations from the linear relationship between whole tissue Cl and sea water Cl which occur at extreme dilutions and extreme concentrations represent the failure of the physiological mechanism to control any longer the integrity of the chloride-free cellular space under relatively drastic conditions.

Investigations of biochemical or physiological changes in tissues of whole organisms, living successfully in controlled foreign environments, allow, in the conclusions drawn, a degree of certainty less frequently assured from data collected on isolated, surviving tissues. In the present instance, it is of much interest to note some close parallelisms between different species investigated by the respective experimental approaches.

The work reported in this paper was begun with the experimental ecological viewpoint in mind. In summarizing briefly, it is recalled that while the adult California mussel is able to adjust itself, with accompanying changes in tissue constitution, to a considerable range of salinities in the laboratory, this species, unlike *M. edulis*, is rarely if ever found in bays and estuaries, even though such waters may be considerably less diluted than were solutions which the animals have been shown experimentally to tolerate for indefinite periods.

Our findings during the course of this work suggested some experiments on the effect of dilute solutions of sea salts upon ripe sperm and

eggs of this species, the process of fertilization, and subsequent development. Some preliminary experiments of this kind were accordingly carried out at the Scripps Institution by Dr. Robert T. Young, whose findings indicate that sea water diluted by more than 25 per cent may exert injurious effects upon (1) the sex products themselves, (2) incidence of fertilization, and (3) subsequent development. Doubtless the dilution of water in bays and estuaries is not the only factor responsible for the failure of *Mytilus californianus* to colonize them, but the sensitivity of eggs, sperm, and larvae to the dilute environments provides one tangible clue which should prove helpful in further attack on this and kindred problems.

Acknowledgments

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Summary

1. Experiments indicate that the adult mussel *Mytilus californiaus* is heterosmotic, yet potentially euryhaline to a considerable degree, although sperm, eggs and larvae are highly stenohaline toward dilution of the environment.

2. The tissue-chloride content is close to 1 per cent by wet weight, varying only slightly with season. Mature males show slightly higher chloride values than do the mature females, due to the higher chloride content of testicular tissues than of ovarian tissues.

3. Mussels can survive for indefinite periods the sudden and continued exposure of their tissues to sea water diluted by 50 per cent (Cl conc. 0.94 per cent) or water concentrated to half again its normal value (Cl conc. 2.73 per cent to 2.8 per cent). Below or above these two respective extremes, sudden immersion is fatal.

4. Within the limits of the physiologically tolerated range indicated, the concentrations of tissue chloride are adjusted to concentrations of chloride in the environment, with maintenance of an approximate value of 1 : 1.60, calculated as grams per 100 ml. of internal and external water.

5. Considerable individual differences exist in the rate of establishment of equilibrium between environmental and tissue chloride concentrations, when mussels are exposed to the indicated dilute and concentrated solutions.

6. While sudden immersion in solutions of sea salts below or above the respective limits resulted fatally, it was possible for mussels to survive in solutions considerably beyond such limits, i.e. in water diluted to as low as 0.62 per cent Cl, or concentrated to 3.48 per cent Cl, if the concentrations were altered by *gradual steps*.

7. Mussels surviving in sea water, gradually diluted to a chlorinity of 0.625 per cent Cl, underwent a fall in their tissue chloride to values of about 0.26 per cent to 0.38 per cent; animals kept in sea water gradually concentrated to a chlorinity of 3.48 per cent underwent a rise in their tissue chloride to average values of 2.25 per cent; at these respective points animals were at their threshold of tolerance and showed incipient sluggishness. Animals of such extreme chloride levels, however, recovered if placed in running sea water, and readily underwent therein a restoration of their tissue chlorides to normal values.

8. Exposure of mussels to the diluted or concentrated solutions results in migrations of both water and chloride between internal and external media.

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MATING REACTIONS OF ENUCLEATE FRAGMENTS IN PARAMECIUM BURSARIA

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INTRODUCTION

The presence of distinct mating types in *Paramecium* has been demonstrated by several investigators (Sonneborn, 1937; Kimball, 1937; Jennings, 1938; Sonneborn, 1938; and Jennings, 1939). Under appropriate conditions individuals belonging to different mating types in the same "group" will, when they are placed together, immediately agglutinate and later form pairs. Such agglutination has been called the "mating reaction." The present study is designed to answer the question: Do enucleate fragments of *Paramecium* manifest mating reactions?

In this investigation we have studied, for comparison, the following phenomena: (1) mating reaction between whole animals, (2) mating reaction between nucleate [1] fragments and whole animals, (3) mating reaction between enucleate [1] fragments and whole animals, (4) mating reaction between enucleate fragments. The results of these comparative studies will be reported in the order given.

Preliminary work on this problem was originally begun at the Osborn Zoölogical Laboratory, Yale University, in collaboration with Dr. R. F. Kimball; but circumstances unfortunately prevented its completion there. The present study was carried on largely at the University of California at Los Angeles and completed at the University of Vermont. A preliminary report appeared in *Science*, **91**: 246 (1940).

MATERIAL AND METHODS

Paramecium bursaria—the green Paramecium—is especially favorable for this study for several reasons. (1) As far as we know, *bursaria* is the only species of *Paramecium* in which enucleate fragments are viable. (2) The tendency of animals of this species to creep slowly over the bottom of the container facilitates cutting with a glass needle to such an extent that large numbers of fragments can be obtained.

[1] In this paper the word "nucleate" is used to indicate the presence of both the macronucleus and the micronucleus; the word "enucleate" meaning the absence of both nuclei.

(3) The single micronucleus of this species (especially in the races used in the present investigation) is large and stains deeply with hematoxylin. (4) Stability of mating type permits one to obtain uniform and constant material for study.

For this study two races of *P. bursaria*—McD_3 and *Gr*14—were used.[2] They belong to two different mating types of Group II (Jennings, 1939). Under suitable conditions they give a marked mating reaction when placed together, and permanent pairs are later formed.

Race McD_3 was collected near Baltimore, Maryland; race *Gr*14 from south of Greensboro, North Carolina. McD_3 is a large race, while *Gr*14 is somewhat smaller. In both races the micronucleus is large and stains deeply with hematoxylin. After such staining the micronucleus or a piece of macronucleus could thus easily be detected if present in any fragment.

The animals were cultured in essentially the same manner as described by Jennings (1939). A number of cultures of each race were kept in the laboratory, and only those which gave the strongest mating reaction were used.

The animals to be operated upon were placed in a depression slide and cut with a fine glass needle under a dissecting microscope. When the needle passed directly through the mid-region of the animal, the nuclei were usually seen to be extruded from one of the fragments; while if the needle cut to one side of the mid-region one large nucleate and one smaller enucleate fragment resulted. Only fragments one-half the size of the original animal or smaller were isolated for testing since these were most likely to be enucleate.

When a definite mating reaction had been observed, the fragments were fixed in hot Schaudinn's fluid (95 cc. Schaudinn's fluid and 5 cc. glacial acetic acid) or in Bouin's fluid at room temperature. They were stained in iron-hematoxylin, destained in aqueous picric acid, and mounted in damar.

Observations

Mating Reaction between Whole Animals

Jennings (1939) has described in detail the mating reaction between whole animals in *P. bursaria*. We have, however, noted an additional feature. When the area of contact of one animal with another is small there appears a distinct flattening if not an appreciable indentation of that part of the cell (Fig. 1). At present an explanation of this phenomenon cannot be given, but at least the response indicates that the

[2] We are indebted to Professor H. S. Jennings for these two races of *P. bursaria*.

union between the animals involves more than a possible adhesion of the

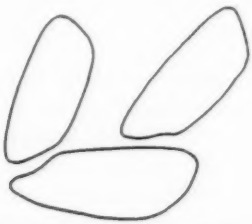

FIG. 1. Mating reaction of whole animals. Two individuals in this case have become attached to a third animal. In each animal there appears a flattening of the contour of the body at the region of contact. (This drawing and others to follow are free-hand sketches from the living material.)

cilia. It is significant that if members of a reacting pair (two whole animals, or a whole animal and a fragment) are gently separated with the glass needle, these flattenings or indentations (Fig. 3, *g–i*) do not disappear at once but only gradually round out to the normal contour.

Mating Reactions between Nucleate Fragments and Whole Animals

When *P. bursaria* is cut transversely with the needle there usually results one larger and one smaller fragment. It is the larger fragment which contains the nuclei, the nuclear complex being visible as a clear sphere in the living fragments. Shortly after cutting, such nucleate fragments of McD_3 were placed with whole animals of *Gr*14. The mating reaction took place at once, and on the following day intimate fusion of fragments with whole animals, similar to conjugation between whole animals, was observed. Conversely, nucleate fragments of *Gr*14 were placed with whole animals of McD_3, and such mixtures gave the same mating reaction and intimate fusion (Fig. 2) as described above.

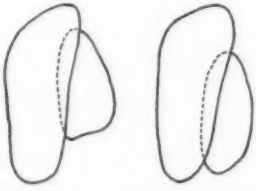

FIG. 2. Large nucleate fragments of *Gr*14 fused with McD_3 whole animals.

(Samples of such pairs were stained with aceto-carmine and a full nuclear complement was invariably found in the fragment.) It has not

yet been determined whether the intimate fusion between the nucleate fragment and the whole animal initiates nuclear changes in either or both partners.

It should also be noted that the nucleate fragments not only exhibited mating reactions immediately but also on the second and third days after cutting. After the mixture was made and mating reaction noted, it was placed aside undisturbed in a moist chamber. This mixture was again examined on the second and third days following. Such nucleate fragments as had not already fused in conjugation were again found to be exhibiting the mating reaction. Apparently the nucleate fragments which did not fuse with the whole animals gave repeated mating reactions.[3] Thus the nucleate fragments can react not only on the day when they are prepared, but also on subsequent days; and this is quite in contrast to enucleate fragments which give the mating reaction on the day of cutting but never subsequently, though they may be active two or more days later (see below).

Viability of Enucleate Fragments

Enucleate fragments of *P. bursaria* may remain alive for as long as four days, and during that time exhibit a surprisingly normal behavior, swimming actively in one direction, alternating the direction of movement, spiralling, and coming to rest adjacent to masses of food (Tartar, 1938). Observations on 100 enucleate fragments each of McD_3 and *Gr*14 showed that practically 100 per cent of such fragments were alive and active after one day, and 50 per cent after two days. Such hardiness of enucleate fragments made possible the investigation herein described. In the present study the enucleate fragments were tested within half an hour after cutting.

Mating Reaction between Enucleate Fragments and Whole Animals

Enucleate fragments of either race were found to give the mating reaction with whole animals of the other race (Fig. 3, *a–f*). Enucleate fragments never agglutinate with fragments or whole animals of the same race. The mating reaction was observed in 131 small enucleate fragments of McD_3 mated with whole animals of *Gr*14. A sample of fifteen of these fragments which reacted was fixed in Schaudinn's fluid and stained with iron-hematoxylin to test whether nuclei or parts thereof might be present in the fragments. In no case was a micronucleus or a piece of macronucleus found in a fragment.

[3] The races studied show a diurnal reactivity, discontinuing mating reactions in the late afternoon and not reacting again until the morning of the following day.

In the reciprocal cross enucleate fragments of *Gr*14 were placed with whole animals of McD_3. Such a mixture gave the same agglutination as described above. Twelve such fragments which reacted were stained and no trace of a nucleus or part of a nucleus was found in them.

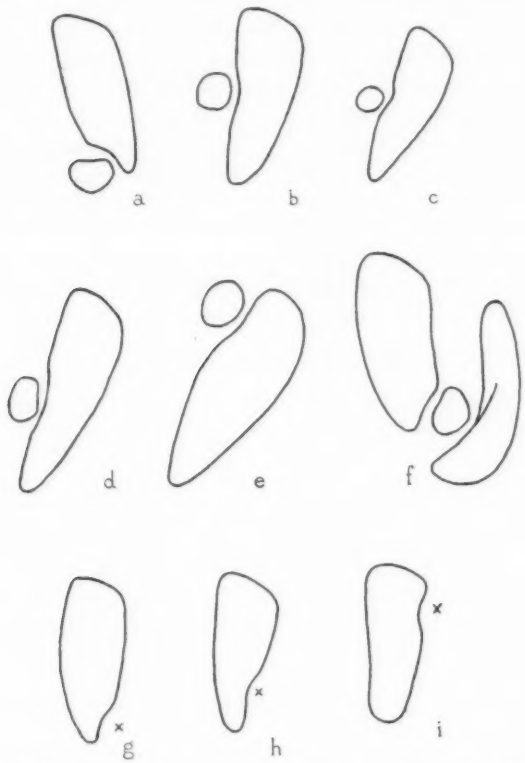

FIG. 3. Mating reactions between enucleate fragments and whole animals. Note the flattening or indentation of the body of the animal at the point of union with a fragment (Fig. 3, *a–f*). Whole animals which were separated from their attachment with enucleate fragments still retain typical indentation (X) at the points of union (Fig. 3, *g–i*).

Apparently in no respect does the mating reaction between enucleate fragments and whole animals differ from that between whole animals. Such similarity is shown by the following observations: (1) The reacting fragment agglutinates with the first animal of the other race with which it happens to collide. Subsequently, one, two, three or more ani-

mals of the other race may become attached to the fragment, thus forming the typical agglutinated clump (Fig. 3*f*). (2) The fragment remains securely attached to the whole animal, i.e., the two do not rotate upon one another. The direct medium of this union is not the surfaces of the fragment and the whole animal since the partners remain separated by the distance of their cilia. Yet the union is more intimate than might be suspected, for at whatever region the fragment attaches to the whole animal, a flattening or slight indentation of the normal contour of the whole animal is there produced (Fig. 3). (3) The pairs or clumps formed by the agglutination of fragments and whole animals break up at approximately the same time as that at which clumps of whole animals mated simultaneously break up into conjugating pairs and single animals. This latter point is the conclusion from a separate study of the duration of agglutination of enucleate fragments with whole animals in which two additional groups of McD_3 enucleate fragments (total number, 90) were mated with *Gr*14 whole animals. All enucleate fragments became separated from the whole animals 123 to 140 minutes after the beginning of agglutination and never again united with them. This was approximately the time required for simultaneously mixed whole animals, which formed large clumps, to break up into pairs and single animals (123 to 153 minutes).

The mating reaction between enucleate fragments and whole animals is thus in these respects altogether normal, ***but it was never followed by true fusion as in conjugation.*** It remains to be determined whether contact with the enucleate fragment is sufficient stimulus to initiate nuclear changes in the whole animals.

Although mating reaction is thus shown by enucleate fragments tested promptly after cutting, this is not the case if the enucleate fragments are kept for 24 hours before they are mixed with whole animals of the other race. Enucleate fragments from a McD_3 culture were prepared at the time when tests showed the animals of this culture to be most reactive with *Gr*14. These McD_3 enucleate fragments were placed with *Gr*14 whole animals 24 hours after they were cut. None of the 115 fragments so tested in delayed mixing showed any mating reaction although they were alive and active. At the time of mixing the enucleate fragments with the whole animals, control experiments showed that the McD_3 whole animals from the culture in question were again strongly reactive.

Mating Reaction between Enucleate Fragments

For a study of reaction between enucleate fragments, one fragment of each race was placed in a very small drop of culture fluid and the

pair observed. In approximately half of the cases the enucleate fragments agglutinated at once when, during their rapid movements, they first collided with one another (Fig. 4). Mating reaction between enucleate fragments was under these conditions apparently not so strong as between enucleate fragments and whole animals, for the pairs were easily separated by jarring, and even when not disturbed they remained attached for not longer than ten minutes. That the response is not a mere chance adherence, however, is shown by the fact that it never occurred between fragments of the same race even though these were observed to collide with one another. Although flattening at the point of contact was not observed in these small fragments, they swam together as one animal without rotating upon one another, a behavior typical of

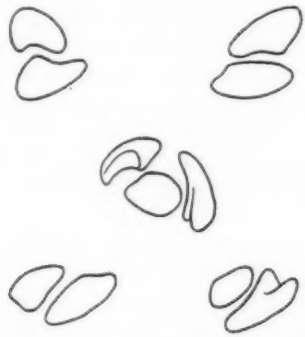

FIG. 4. Mating reaction between enucleate fragments. (These fragments were later fixed and stained and were found to be enucleate.)

the mating reaction. After the two reacting fragments separated, they frequently again agglutinated and became attached.

Thirty enucleate fragments (15 of each race) were tested as described; and of these 16 gave the mating reaction. Each pair of reacting fragments was separately fixed and stained in iron-hematoxylin. In no case was a nucleus or part of a nucleus found in any of the fragments.

Another method of observing the mating reactions between enucleate fragments consists of introducing under the dissecting microscope one fragment of one race into a drop containing several fragments of the other race and to watch the introduced fragment continuously. This procedure greatly increases the probability of the fragment finding a partner. Under these circumstances two more cases of mating reaction were observed: in one case the introduced McD_3 fragment remained

attached to two *Gr*14 fragments for five minutes; in the other case a McD_3 fragment paired with a *Gr*14 fragment for twelve minutes.

Still another method was to place three to six enucleate fragments of one race (*Gr*14) into a drop containing many enucleate fragments of the other race (McD_3). Out of a total of 22 *Gr*14 fragments so tested, 15 reacted. Typical mating reaction occurred; pairs of enucleate fragments and groups of three enucleate fragments were observed. When, as here, the drop of water in which the reaction is followed is of sufficient size that evaporation does not interfere and the reacting fragments need not be disturbed by replenishment of the drop, the conditions may be said to be most nearly normal; and under these circumstances pairs of enucleate fragments and groups of three enucleate fragments were found to remain continuously united in the mating reaction for as long as 34 minutes.

Thus a total of 25 cases of mating reaction between enucleate fragments has been recorded. Of these, 12 sample pairs of fragments were carefully stained and no nucleus found in either fragment. In every experiment, it is to be emphasized, the unmixed enucleate fragments of each race were conscientiously watched en masse, and in no instance did fragments of the same race react with one another though collisions between them were frequent.

Thus the cytoplasm alone (in the absence of the nuclei) exhibits the reactivity and diversity of mating type. Of course, this reactivity may be due to the retention of influence of the nuclei which have just been removed. This possibility is strongly suggested by the fact that enucleate fragments lose their reactivity within a day and do not regain it thereafter.

Summary

1. Comparative studies were made on the following phenomena in two races of *Paramecium bursaria* belonging to two different mating types: (*a*) the mating reaction between whole animals, (*b*) the mating reaction between nucleate fragments and whole animals, (*c*) the mating reaction between enucleate fragments and whole animals, and (*d*) the mating reaction between enucleate fragments.

2. In the mating reaction between whole animals a phenomenon hitherto unreported was observed: When two animals become attached there is a flattening or even an appreciable indentation at the point of union which is most striking when the area of contact is small.

3. Nucleate fragments of either race show mating reaction with whole animals belonging to the other race. The mating reaction may be followed by an intimate fusion, as in conjugation between whole animals, between the nucleate fragment and whole animal.

4. Enucleate fragments of either race give the mating reaction with whole animals of the other race. Mating reactions never occur between enucleate fragments and whole animals of the same race. Mating reaction between enucleate fragments and whole animals appears to be identical with that between whole animals, but it was never followed by intimate fusion as in conjugation between whole animals.

5. The mating reaction also occurs between enucleate fragments belonging to two different races. Controls showed that mating reactions never occur between enucleate fragments of the same race.

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